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Reparación de daño cerebral mediante biomateriales y terapia celular: estudio experimental
Brain damage repair through materials and cell therapy : experimental study

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REPARACIÓN DE DAÑO CEREBRAL MEDIANTE BIOMATERIALES Y TERAPIA CELULAR. ESTUDIO EXPERIMENTAL.

BRAIN DAMAGE REPAIR THROUGH BIOMATERIALS AND CELL THERAPY. EXPERIMENTAL STUDY.

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A mis padres,

por ser mi faro en los días de galerna.

AGRADECIMIENTOS

"*Ars longa, vita brevis*"

Ésta es una frase que pronunció Hipócrates, considerado el padre de la medicina (460-370 a.C.), y viene a decir que el arte es largo y la vida breve.

No hay frase que defina mejor la sensación que he tenido estos últimos meses, a la hora de recapitular el trabajo realizado, siendo consciente de todo lo que queda por hacer, y alarmándome de lo rápido que ha pasado el tiempo.

En este viaje, es momento de reflexión. De mirar atrás y recordar lo vivido. De volver a esa primera vez que entré en aquel despacho. Era una mañana de septiembre y con tan sólo una corta conversación, Juan Antonio decidió confiar en mí. Se ha llegado a convertir en mi mentor, mi tutor y mi guía, incluso en mi consejero en algunas situaciones. Con argumentos y experiencia, sugiere y recomienda, sin tener la necesidad de imponerse. De él, he aprendido no sólo ciencia, sino a pensar por mí misma.

Y así como uno responde al *Qué*, el *Cómo* lleva aire mejicano. Ha sido de él, Ulises, de quien he aprendido no sólo protocolos, si no a saber buscar y no parar hasta encontrar la letra pequeña y los detalles, que marcan la diferencia.

En esa misma mañana conocí el Laboratorio, esas paredes de azulejos que me han visto llorar y reír, aprender y equivocarme, y en definitiva, evolucionar. Esa bonita palabra de tan difícil digestión. Esas personas sencillamente maravillosas son las que responden al *Quien*. Algunas se han ido, otras están y otras se irán, pero no olvidaré cada granito que me han aportado. Palabras de ánimo, de aliento, de alegría; penas compartidas y alguna que otra cerveza de más. En estas páginas, hay pequeños pedacitos de ell@s, y si lo he conseguido, ha sido por un trabajo en equipo.

Marisol, sinónimo de estabilidad y templanza, ayuda sin invadir y participa sin hacer ruido. Es de esas personas que deja que brille el de al lado, sin darse cuenta de la luz propia que ella desprende.

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A velocidad de crucero y con ideas fijas a pesar de los serpenteos del rio, así es ella. Lidia siempre tiene tiempo para un café, haciéndote sentir que no libras la batalla sola.

Y a medio camino entre los quirófanos y el animalario, entraron en escena ellos, los veterinarios. Dos personas muy diferentes entre sí que forman un gran equipo. Pablo, de ti he aprendido a escuchar además de oír. Ahora entiendo las historias que nos contabas, lecciones que marcan el camino y no deben decirse a voces. Y Cruz, con alma hippie, consigue dividirse y estar en dos sitios a la vez. Con una sonrisa, siempre atiende tus plegarias, que generalmente son urgentes además de importantes.

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Y aunque no lo diga en alto tantas veces como debería, no habría podido vivir esta aventura sin ellos: mis padres, las personas que más se alegraron aquella mañana de septiembre. Responden al *Cuándo* y lo dijeron alto y claro: ahora. Todavía me sigo asombrando de su forma tan incondicional y desinteresada de querer. Han sabido ser mi equilibrio: cautelosos cuando estaba exaltada y positivos cuando estaba triste.

Y dentro del núcleo familiar esta ella, una pequeña vocecita que, aunque no suelo oírla a menudo, cuando lo hago, retumba en mis adentros. Si yo digo negro, ella blanco, y viceversa. Todavía la debo un bolazo de nieve. Relación de hermanas supongo.

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Lejos pero cerca, está el resto de mi familia. Abuelos, tíos y primos, con los que comparto ruidosas navidades llenas de alegría y confeti. Sonrío al ver crecer a los más pequeños, pero aún sonrío más con las canas de los mayores, que nunca pierden su sentido del humor. Todos ellos son para mí, bocanadas de aire norteño.

Por último, y no por ello menos importante, está la figura más silenciosa de todas, Vir. Aguanta estoicamente los vaivenes de mis tempestades, preparada para lo que pueda venir. Encuentra las palabras para hacerme reír y crear la calma que necesito. Pero resulta que esa calma en la que me gusta acurrucarme está llena de pelos. Unos blancos y largos, y otros negros y cortos. A los dos leones de la casa no les hace falta hablar, porque ya lo dicen todo. Juntos, formamos un equipazo.

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ABREVIATURAS

AIT	Ataque Isquémico Transitorio
Ag	Antigeno específico de superficie
Ang-1	Angiopoyectina 1
ASCs	Células madre mesenquimales de origen adiposo
BDNF	Factor neurotrófico derivado del cerebro
BHE	Barrera hematoencefálica
BM	Biomaterial
BrdU	Bromodesoxiuridina
CBV	Volumen sanguíneo cerebral
CD31	Molécula de adhesión de células endoteliales plaquetarias
DCX	Doblecortina
ECV	Enfermedad Cerebrovascular
EP	Enfermedad de Parkinson
FGF	Factor de crecimiento fibroblástico
GAGs	Glucosaminoglucanos
GFAP	Proteína ácida fibrilar glial
HA	Ácido hialurónico
HAS	Enzimas hialuronano-sintasas
HFG	Factor de crecimiento de hepatocitos
Human Nuclei	Human specif nuclei
Iba1	Molécula adaptadora de unión a calcio ionizada 1
ISCT	Cell Committee of the International Society for Cell and Gene Therapy
LCR	Líquido cefalorraquídeo

L-DOPA	L-3,4 dihidroxifenilalanina
MSCs	Células madre mesenquimales
NF160kD	Neurofilamentos inmaduros
NF200kD	Neurofilamentos maduros
OEG	Glía envolvente olfatoria
OMS	Organización Mundial de la Salud
PCL	Policaprolactona
PDGF	Factor de crecimiento derivado de las plaquetas
PLGA	Ácido láctico-co- glicólico
QALY	Años de Vida Ajustado por Calidad
r-tPA	Activador Tisular de Plasminógeno Recombinante
SC	Células de Schwann
SNC	Sistema nervioso central
SNP	Sistema nervioso periférico
SNS	Sistema Nacional de Salud
SVZ	Zona Subventricular
TH1	Tirosina hidroxilasa
VEGF	Factor de crecimiento endotelial vascular
VRM	Vía rostral migratoria

RESUMEN

La enfermedad cerebrovascular es la primera causa de discapacidad en el mundo occidental. A menudo, ésta y otras patologías del sistema nervioso central cursan con pérdida funcional de neuronas y células gliales. Los tratamientos actuales no dan respuesta a las secuelas desencadenadas y es preciso desarrollar estrategias terapéuticas innovadoras con el objetivo de conseguir una mejora funcional de los pacientes. La terapia celular, en gran expansión ésta última década, ha demostrado ser una alternativa eficaz, según datos pre-clínicos, en este ámbito.

El objetivo de la utilización de terapia celular, en nuestro caso, células madre mesenquimales derivadas de tejido adiposo, en combinación con un biomaterial tridimensional y poroso de ácido hialurónico es para alcanzar un efecto sinérgico. Este efecto se consigue gracias al carácter biocompatible, biodegradable y a su capacidad de albergar células exógenas y endógenas, del biomaterial una vez implantado. Ha sido demostrado el efecto antiinflamatorio, modulador, y quimio-atrayente de las células madre mesenquimales, las que no se han integrado ni diferenciado de forma espontánea en el tejido hospedador. Su papel de bomba trófica se potencia si su liberación es en la diana terapéutica, y aún más, si se administran en el interior de un andamio (*scaffold)* que, por un lado evita su dispersión en el parénquima, y por otro, actúa de soporte estructural de células endógenas que llegan al lugar de lesión.

Hemos obtenido unos resultados prometedores, habiendo observado una completa integración del biomaterial en el tejido; una reducción de la cicatriz astrocitaria y de la reacción glial en los grupos con el biomaterial y las células en comparación con el grupo contro. Además, hemos encontrado un mayor número de células BrdU positivas en la zona subventricular ipsilateral, lo que corrobora el efecto sobre la neurogénesis de las células. En paralelo, las imágenes de resonancia magnética no sólo corroboran la biocompatibilidad e integración del biomaterial; sino que además, es posible comparar el volumen de lesión isquémica entre los distintos grupos. Existe una diferencia volumétrica estadísticamente significativa entre los tres grupos,

observando una reducción de la lesión en el gurpo con biomaterial y células respecto del control.

Además, hemos desarrollado un *scaffold* de policaprolactona capaz de guiar el crecimiento axonal entre dos regiones cerebrales distantes, potenciado por el efecto de células de la glía olfatoria envolvente. Esta estrategia transversal aplicada múltiples patologías que cursen con pérdida de la conectividad axonal, tiene especial relevancia en la enfermedad de Parkinson; en la cual, la reconexión axonal podría tener un papel protagonista.

En este caso, hemos observado un mayor número de marcaje TH positivo en el interior del andamio y una mayor densidad de fibras en el interior del biomaterial con células de la glía olfatoria envolvente respecto del grupo con el biomaterial.

En conclusión, los *scaffolds* utilizados son biocompatibles, y capaces de albergar células tanto exógenas como endógenas en su interior. Las células madre mesenquimales favorecen la neurogénesis endógena, reducen la reacción glial y la cicatriz astrocitaria. Además, su administración en el interior de un andamio de ácido hialurónico tiene un efecto sinérgico, consiguiendo reparar parcialmente la lesión y reducir el volumen isquémico. Por otro lado, el scaffold de policaprolactona guía el crecimiento axonal, conectándo así dos zonas distantes en el SNC; y las células de la glía olfatoria envolvente promueven y facilitan la re-entrada de los axones del interior del biomaterial en el parénquima.

SUMMARY

Cerebrovascular disease is the leading cause of disability in the Western world. Often, this and other pathologies of the central nervous system present with functional loss of neurons and glial cells. Current treatments do not respond to the sequelae triggered and it is necessary to develop innovative therapeutic strategies with the aim of achieving a functional improvement in patients. Cell therapy, in great expansion during the last decade, has proven to be an effective alternative, according to pre-clinical data, in this field.

The objective of the use of cell therapy, in our case, mesenchymal stem cells derived from adipose tissue, in combination with a three-dimensional and porous hyaluronic acid biomaterial is to achieve a synergistic effect. This effect is achieved thanks to biocompatible, biodegradable character and its capacity to house exogenous and endogenous cells, of the biomaterial once implanted. The anti-inflammatory, modulatory, and chemo-attractive effect of mesenchymal stem cells has been demonstrated, which have not been integrated or differentiated spontaneously in the host tissue. Its role as a trophic pump is enhanced if its release is in the therapeutic target, and even more so, if they are administered inside a scaffold that, on the one hand, prevents its dispersion in the parenchyma, and on the other, acts as a structural support for endogenous cells that reach the site of injury.

We have obtained promising results, having observed a complete integration of the biomaterial in the tissue; a reduction of the astrocytic scar and of the glial reaction in the groups with both the biomaterial and the cells in comparison with the control group. In addition, we have found a greater number of BrdU positive cells in the ipsilateral subventricular zone, which corroborates the effect on cell neurogenesis. In parallel, the magnetic resonance images not only corroborate the biocompatibility and integration of the biomaterial; but also, it is possible to compare the volume of ischemic injury between the different groups. There is a statistically significant volumetric difference between the three groups, observing a reduction of the lesion in the group with biomaterial and cells with respect to the control.

In addition, we have developed a polycaprolactone scaffold capable of guiding axonal growth between two distant brain regions, enhanced by the effect of enveloping olfactory glia cells. This cross-sectional strategy applied to multiple pathologies that occur with loss of axonal connectivity, has special relevance in Parkinson's disease, in which axonal reconnection could have a leading role.

In this case, we have observed a greater number of positive TH marking inside the scaffold and a higher density of fibers inside the biomaterial with olfactory glia ensheathing cells with respect to the group with the biomaterial.

In conclusion, the scaffolds used are biocompatible, and capable of harboring both exogenous and endogenous cells in their interior. Mesenchymal stem cells favor endogenous neurogenesis, reduce glial reaction and scar astrocyte. In addition, its administration inside a hyaluronic acid scaffold has a synergistic effect, partially repairing the lesion and reducing the ischemic volume. On the other hand, the polycaprolactone scaffold guides axonal growth, thus connecting two distant areas in the CNS; and enveloping olfactory glia cells promote and facilitate re-entry of the axons inside the biomaterial into the parenchyma.

ESTADO DEL ARTE

La **enfermedad cerebrovascular** (ECV) o accidente cerebrovascular se refiere a todo trastorno en el que un área del encéfalo se afecta de forma transitoria o permanente por una isquemia o hemorragia, estando uno o más vasos sanguíneos cerebrales afectados por un proceso patológico.[1]

La Organización Mundial de la Salud (OMS) define el accidente cerebrovascular como "*el desarrollo rápido de síntomas clínicos indicativos de un trastorno local o generalizado de la función cerebral, con síntomas que persisten 24 o más horas o que conducen a la muerte sin que exista causa aparente otra que la vascular*".[2]

Según los datos de la Sociedad Española de Neurología, a nivel nacional, el ictus es la primera causa de mortalidad en mujeres y la segunda en hombres; constituyendo además la primera causa de discapacidad en mayores de 65 años. Aproximadamente, entre el 25-30% de los supervivientes a un accidente cerebrovascular, desarrollarán deterioro cognitivo o demencia.[3] Debido a la importancia epidemiológica y al gran gasto socioeconómico que supone, es prioritario avanzar en su prevención, control y tratamiento. Esta elevada tasa epidemiológica es debida, en gran medida, a un gran listado de **factores de riesgo**, que incluye edad, sexo, factores genéticos, psicosociales y hábitos de vida. La importancia de la identificación de éstos radica en la **prevención** de la enfermedad; siendo un ejemplo de ello el estudio *Interstroke*.[4]

Según su naturaleza, se puede distinguir entre **ictus hemorrágico e ictus isquémico**. El ictus hemorrágico comprende aproximadamente el 10-15% de los casos, y es causado por la presencia de sangre en el parénquima cerebral, en el interior de los ventrículos cerebrales o en el espacio subaracnoideo. En cambio, el ictus isquémico (denominado también isquemia focal) corresponde al 85-90% de la casuística y es producido por una interrupción del aporte sanguíneo. Dependiendo de la duración del trastorno, las ECV se clasifican en **ataque isquémico transitorio** (AIT), con una duración menor de 24 horas, de carácter reversible y no produciendo déficits neurológicos; o en

***ictus*,** cuando el proceso se prolonga durante más de 24 horas, produciendo secuelas neurológicas.[5]

La elevada incidencia del ictus isquémico acompañado de los datos de QALY,[6] que se define como los años de vida ajustados por calidad que presentan los pacientes, le convierten en una prioridad sociosanitaria.

El principal problema del evento isquémico radica en la restricción o reducción del aporte de oxígeno, glucosa y nutrientes en el área afectada. El área totalmente privada de aporte sanguíneo es denominada **núcleo o *core*** del ictus y, en términos terapéuticos, es un área irreversible/insalvable. No obstante, existen regiones próximas al núcleo que han tenido acceso a una circulación sanguínea colateral, siendo capaces de contrarrestar parcialmente el déficit energético, denominada **zona de penumbra**.[7-8]

Por añadidura, el cuadro se agrava por particularidades del sistema nervioso central (SNC): los depósitos de glucosa y glucógeno del cerebro tan sólo son capaces de cubrir los requerimientos energéticos cerebrales durante un minuto y el carácter selectivo de la barrera hematoencefálica (BHE) limita la velocidad de transferencia de moléculas desde el torrente sanguíneo al cerebro, restringiendo el acceso a substratos necesarios para el metabolismo celular.[9]

Es importante recalcar que la **cascada isquémica** se inicia mientras existe obstrucción arterial. La muerte celular accidental de las células del *core* va acompañada de eventos de excitotoxicidad por glutamato, el estrés oxidativo y la neuroinflamación, que afectan al funcionamiento homeostático de las neuronas existentes en el área afectada. La combinación de todos ellos induce la activación de vías de muerte celular y la generación de lesiones cerebrales permanentes.[10-12]

Sin embargo, en el momento en que la **obstrucción arterial remite**, tiene lugar la reperfusión del parénquima cerebral y la propia reanudación de acceso a oxígeno agrava la cascada isquémica ya iniciada.[7,8]

El factor **tiempo** es determinante para salvar la mayor cantidad de tejido cerebral en el área de penumbra. A pesar de las **respuestas compensatorias**, entre las que destacan la recuperación parcial del metabolismo oxidativo y el intento de estabilización del flujo sanguíneo; se mantiene la tríada de signos característica de la lesión hipoxia-isquemia: consolidación de excitotoxicidad dependiente de glutamato, estrés oxidativo y neuroinflamación aguda.[7,8,13]

El periodo de tiempo en el que es posible **reducir el impacto** del daño hipóxico-isquémico, conocido como **ventana terapéutica**, en la zona de penumbra es de entre 1 a 6-24 horas. En esta fase, es decisiva la restauración del flujo sanguíneo cuanto antes para minimizar la extensión de la lesión cerebral.[13]

A pesar del drástico aumento de casos en los últimos años, se ha registrado una disminución de la tasa de mortalidad gracias a los esfuerzos de prevención, detección precoz y mejora de la atención especializada hospitalaria. El control de factores de riesgo y la implantación de las ***Unidades de Ictus*** en el Sistema Nacional de Salud (SNS) han sido las claves del éxito. Estas Unidades especializadas han implementado protocolos de tratamiento unificados y un riguroso control de las constantes vitales de los pacientes.[14,15]

Actualmente, los tratamientos autorizados para esta patología se reducen a la eliminación mecánica del trombo mediante una **trombectomía** y/o a su disolución química mediante un fármaco trombolítico denominado activador tisular del plasminógeno recombinate (**r-tPA**). A pesar de que este fármaco es el tratamiento más utilizado durante la fase aguda, cuenta con grandes limitaciones relacionadas con los criterios de uso (ventana terapéutica <4,5h, fenómenos de extravasación, enfermedades comórbidas, edad etc.).[16-18]

Ensayos clínicos a nivel mundial están desarrollando estrategias de **neuroprotección** enfocadas a reducir el daño en el área de penumbra para su uso en combinación con uno o más de los tratamientos terapéuticos autorizados.[19]

La duración de la fase subaguda es de entre 6-24 horas a días desde el accidente isquémico; y en los casos en los que el manejo de la patología no haya sido el adecuado, será característico una hipoperfusión y un mantenimiento del mecanismo de *feed-back* positivo de la tríada (excitotoxicidad, estrés oxidativo e inflamación). En consecuencia, aumenta la extensión del área de penumbra y comienza el fenómeno de *gliosis* por la presencia de astrocitos reactivos.[10,13]

A pesar de los eventos patológicos mantenidos, hay evidencias científicas de la activación espontánea de procesos endógenos de **reparación y repoblación** del área dañada, mediante la **neurogénesis** endógena.[20,22]

La **neurogénesis** se define como el proceso por el cual se forman nuevas neuronas a partir de precursores, localizados en zonas específicas conocidas como nichos neurogénicos , desde donde migran, se diferencian y se integran en su destino hasta convertirse en neuronas funcionales.[23]

A pesar de que la zona subventricular (SVZ) no es el único nicho neurogénico en el cerebro adulto, es la principal fuente de repoblación de neuroblastos a la zona isquémica. El aumento transitorio y **espontáneo** de progenitores, es producido por un acortamiento del ciclo celular, comenzando a los dos días y alcanzando el máximo en dos semanas tras el inicio del daño retornando a sus niveles basales a las seis semanas tras el mismo.[24-27]

Además de la estimulación de la proliferación en SVZ, se ha descrito que los neuroblastos, que de forma fisiológica **migran** por la vía rostral migratoria (VRM) hasta el bulbo olfatorio, son redirigidos hacia el área lesionada.[21-23] La migración ectópica comienza a los 3 o 4 días desde el daño isquémico y se mantiene hasta 4 meses después

del mismo. La redirección se produce por estímulos enviados desde la zona isquémica mediante dos vías: a través de cambios en la composición del líquido cefalorraquídeo (LCR) o a través de la difusión de señales por los vasos sanguíneos.[20,28]

A pesar de los mecanismos desencadenados por el propio organismo para restaurar la zona dañada, son muy pocos los precursores que consiguen alcanzar el área diana; y menos aún, madurar y repoblar la zona.[22] Este fracaso puede ser debido al ambiente inflamatorio[29], al déficit de las conexiones funcionales y de soporte trófico necesario.[30]

Aquellos pacientes que sobreviven a la fase aguda del ictus isquémico entran en la fase crónica de la enfermedad, experimentando una lenta mejoría conocida como **neuroreparación.** El flujo sanguíneo se estabiliza, se interrumpen procesos patológicos celulares como el estrés oxidativo y comienzan procesos reparación vascular; que se suman al proceso de neurogénesis espontánea ya activado.[27]

En los últimos años, datos obtenidos de imágenes en resonancias magnéticas en modelos experimentales de daño isquémico han revelado procesos de **remodelación vascular** tanto aguda como crónica, observando un aumento del volumen sanguíneo cerebral (CBV) en su fase tardía inducido por la estimulación espontánea de la **angiogénesis**. Son los factores angiogénicos secretados por el propio sistema nervioso los que juegan un papel clave en la migración de células endoteliales, la identidad y el crecimiento celular y la regulación de la BHE; estando involucrados, también, en la alineación de vasos-nervios y nervios-arteria en el cerebro. [31-33] Por tanto, el proceso de la angiogénesis se ha postulado como un mecanismo restaurador clave en la respuesta a un evento isquémico que participa en la recuperación funcional después del accidente cerebrovascular.

Además de los mecanismos de neurogénesis y angiogénesis anteriormente descritos, existen otros mecanismos de reparación endógena condicionados por la **anatomía y las particularidades del SNC**. Las neuronas no se renuevan periódicamente

como otras células del organismo y son especialmente sensibles ante cualquier variación anómala en su metabolismo.[29] Cuando existe un daño axonal, los oligodendrocitos se retraen y producen, junto con la propia neurona, un cambio en la composición química del medio extracelular. Esta alteración sirve de estímulo quimiotáctico para la microglía, por un lado, y para los astrocitos por otro.

Las **células gliales** cambian su morfología, y alteran el pH del medio.[34, 35] Adoptan un comportamiento que, paradójicamente, actúa en detrimento de la auto-regeneración de las neuronas afectadas, ya que producen una respuesta inflamatoria exacerbada, que puede generar disfunciones en el tejido sano circundante.[36] Además, requieren un periodo largo para fagocitar y degradar los desechos de células muertas, por lo que la recuperación de la función neural depende, casi en exclusiva, de la capacidad de las neuronas cercanas no afectadas para generar nuevas sinapsis, lo que se conoce como **plasticidad neuronal**.[37, 38]

Los **astrocitos** se activan, modificando así su fenotipo (astrocitos reactivos) para expresar una serie de factores inhibitorios, convirtiendo así la zona dañada en una región de tránsito restringido para el cono de crecimiento axonal.[37,36,39] Además, dichos astrocitos comienzan a sintetizar grandes cantidades de proteoglicanos de condroitín sulfato, formando una membrana fibrosa, conocida como **cicatriz glial**, que actúa como barrera física.[36,37,39,40] Esta rápida reacción de la microglía y de los astrocitos tiene como fin contener el daño y evitar que éste se propague, sellando rápidamente la vía abierta a través de la BHE y evitar que el daño se extienda.

La regeneración axonal está muy limitada en el SNC, lo que se ve acrecentado en patologías donde los tractos de sustancia blanca están involucrados: traumáticas, vasculares o neurodegenerativas. Este defecto se debe, probablemente, a la carencia de factores quimio-atrayentes o a la presencia de elementos inhibidores en el SNC (como la barrera astrocitaria), a diferencia del sistema nervioso periférico que posee una notable capacidad de regeneración.[40]

Por si no fuera suficiente, cuando existe una alteración idiopática, se ve reducido el efecto beneficioso de las respuestas que influyen positivamente en la regeneración autónoma. Este es el caso de la **enfermedad de Parkinson** (EP), donde se forman inclusiones citoplasmáticas llamadas cuerpos de *Lewy* en el soma de neuronas dopaminérgicas de la sustancia negra (el núcleo subcortical) y en células de alrededor,[41] causando la interrupción metabólica de las células y apoptosis. De esta manera, los axones degeneran y se reduce drásticamente la liberación de dopamina en el núcleo estriado, el principal blanco de las sinapsis del tracto nigroestriatal.[41]

A pesar de todos los mecanismos de reparación endógena, persiste la gliosis y la inflamación en la zona de penumbra se **cronifica,** dificultando las estrategias endógenas de reparación[38].

Este es el momento perfecto para implantar estrategias que potencien los mecanismos de reparación endógena y de plasticidad neuronal, con el fin de reconstruir parcialmente el tejido dañado y restaurar la función neurológica. Además del enfoque regenerativo del ambiente neuronal, debemos incidir también en restaurar los tejidos de soporte como los vasos sanguíneos.[8,12,31,32]

Uno de los programas consolidados para fomentar estos mecanismos es la **rehabilitación** que tiene dos objetivos fundamentales; en primera instancia busca reducir o minimizar los déficit o discapacidades del paciente, y en segunda instancia, trata de ayudarle a adaptarse a ellos porque existe la posibilidad de que la recuperación neurológica sea parcial; en función de la gravedad del accidente isquémico. Es un proceso activo que requiere la colaboración y capacidad de aprendizaje del paciente.[1,3]

Los programas de rehabilitación consisten fundamentalmente en la aplicación de técnicas de terapia física, ocupacional y logopedia, según el tipo de discapacidad. Pueden ser utilizadas aisladamente o en combinación con otras técnicas como, por ejemplo, técnicas de neuro-facilitación neuromuscular o técnicas de *bio-feedback*. Ha

sido demostrado que la combinación de todas estas terapias incrementa los procesos de reparación endógena, fundamentalmente la neurogénesis.[3]

Otra de las estrategias más prometedoras es la **medicina regenerativa** es un campo transversal que tiene como fin la estimulación de procesos fisiológicos de regeneración de los tejidos adultos. Induce la reparación y la autoregeneración de tejidos y órganos lesionados que han perdido su estructura funcional, por procesos patológicos o por envejecimiento.[42]

Se sirve de tres estrategias destacadas: **terapia celular, ingeniería tisular** y factores de crecimiento. Las células aportan soporte trófico, mientras que el *scaffold* proporciona el entorno estructural adecuado para que las células puedan realizar sus funciones de manera efectiva. Existen diversos factores críticos involucrados en esta táctica regenerativa, que incluyen la fuente celular, la composición y diseño del *scaffold* y los modelos animales adecuados.[43]

La terapia celular implica la utilización de células vivas para restaurar, mantener o mejorar la función de tejidos y órganos. Su objetivo es el reemplazo de células enfermas o dañadas por otras nuevas funcionalmente activas.[44] Las células madre adultas son las que conducen a la producción y mantenimiento de la mayoría de los linajes celulares en el organismo adulto, mediante de procesos de proliferación, migración, diferenciación y maduración celular.[45]

La utilización de **células madre mesenquimales (MSCs**) se ha popularizado en la comunidad científica en las últimas dos décadas. Estas células son de origen mesodérmico, con propiedades multipotenciales y morfología fibroblastoide. Además de su capacidad de autorrenovación ilimitada, pueden diferenciarse en diversos tipos de células, incluyendo osteocitos, condrocitos, adipocitos, hemocitoblastos, mastocitos y fibroblastos.[46-48]

Cell Committe of the International Society for Cell and Gene Therapy (ISCT) propuso tres criterios comunes para definir las MSC humanas tanto para investigaciones científicas básicas como para estudios preclínicos: adherentes al plástico, expresión de antígeno específico de superficie (Ag) y potencial multipotencial de diferenciación. El objetivo de esta declaración es proporcionar a la comunidad científica un conjunto de estándares para definir la identidad de las MSC.[49]

Concretamente, **las células madre mesenquimales de origen adiposo** (ASCs) se han convertido en los últimos 10 años en una de las principales herramientas de la terapia celular. Es frecuente obtenerlas de lipoaspirados, habiendo variaciones de concentración en función de la zona de obtención.[50] De entre los mecanismos de acción de las ASCs, es su acción trófica la que actualmente se está explorando. A través de la secreción de moléculas de señalización son capaces de generar un microambiente favorable para la reparación de tejidos. Además, actúa como inmunorregulador de forma directa o a través de mediadores moleculares en las zonas lesionadas. Por último, las ASCs secretan factores de crecimiento como HFG, VEGF, PDGF, FGF que activan la proliferación de las células endógenas. Por tanto, presentan ventajas que las hace idóneas para su aplicación: fácil manejo, amplia disponibilidad para su uso alogénico y sencilla extracción, manteniendo su capacidad de autorenovación.[51-55]

Estudios experimentales han utilizado injertos de nervios periféricos y células de Schwann (SC) para promover la reparación en el SNC, dado el éxito en el sistema nerviosos periférico (SNP).[56-57] Sin embargo, esta estrategia no ha sido totalmente exitosa y ha sido necesario buscar mediadores de reparación más efectivos.[58] Durante los últimos años, la **glía envolvente olfatoria (OEGs)** ha tenido una atención especial debido a sus prometedores resultados *in vivo*.[59,60] El trasplante de esta población de células gliales tiene como objetivo fomentar mecanismos de neuroprotección, regeneración axonal y plasticidad.[61]

Estas células proporcionan un entorno favorable para el desarrollo de los axones sensoriales de la mucosa olfativa y guían a éstos hasta el bulbo olfatorio.[62-64] Son

capaces de envolver a los axones para aislarlos del ambiente, evitando que entren en contacto con factores inhibitorios de crecimiento axonal presentes en el SNC. Secretan moléculas de adhesión que promueven el crecimiento axonal[65], neurotrofinas y otros factores tróficos, responsables de la extensión de las neuritas.[66] Del mismo modo, pueden producir citoquinas implicadas en la neuroprotección y la reparación.[67]

Un fenómeno de gran importancia de las OEGs es que son capaces de interactuar con los astrocitos y las células de las meninges, al contrario que las SC. La interacción OEG-astrocito implica un cambio en la cicatriz glial, gracias a su capacidad para penetrar en el tejido de la brecha; esto puede ser un evento crucial para la regeneración de los axones dentro del área de la lesión.[56,67]

La **ingeniería tisular** es un campo interdisciplinar donde se combinan conocimientos de bioingeniería con biología celular y molecular para regenerar o reparar tejidos funcionales. Se desarrolla una **matriz extracelular** transitoria o permanente, que facilita el crecimiento celular hasta formar, de nuevo, la estructura tridimensional del tejido u órgano que se quiere regenerar.[68-72]

La característica más relevante que debe tener un biomaterial o matriz para ser utilizado en ingeniería tisular es su **biocompatibilidad** con el tejido, es decir, ser biológicamente aceptado por el tejido hospedador, y que además, sea capaz de mantener sus prestaciones durante su vida útil.[73] Producen una mínima respuesta inmune e inflamatoria y permanecen estables en la localización implantada permitiendo así la reparación del tejido.[74]

Los biomateriales más versátiles son los polímeros **biodegradables**, capaces de mantener la estructura hasta que el nuevo tejido formado sustituya al soporte. Son degradados en el propio medio biológico y eliminados por la actividad celular.[75,76]

La elevada **porosidad** favorece la acomodación y adherencia de células, y la presencia de poros interconectados facilita su distribución uniforme; y, por tanto, la difusión de oxígeno y nutrientes.[77,78]

El **ácido hialurónico** (HA) es un glicosaminoglicano que se encuentra de forma natural en el tejido conectivo de la mayoría de los vertebrados. Las encargadas de su formación y, por tanto, de su tamaño y peso molecular son las enzimas hialuronano-sintasas (HAS);[79] y se encuentran en la membrana celular de fibroblastos, queratinocitos, condrocitos y células especializadas de tejido conectivo. El peso molecular del HA está directamente relacionado con sus funciones biológicas; al del peso ≥60kDa se le atribuyen propiedades antiadherentes para las células,[80] inhibiendo así la formación de cicatriz glial y manteniendo a los astrocitos en un estado quiescente y no reactivo.[81,82,83]

Recientemente, han evidenciado la existencia de una matriz extracelular en el tejido nervioso, llegando a componer entre el 15 – 25% del tejido. Está compuesta por HA, además de otros glucosaminoglucanos (GAGs) y proteoglucanos. Se encuentra rodeando el cuerpo y las dendritas de neuronas, principalmente interneuronas. También es conocida como red perineural y aunque todavía se desconocen todas sus funciones, interviene en procesos de desarrollo (migración celular, crecimiento y guía de axones y dendritas), formación y estabilización de las sinapsis, mantenimiento de la homeostasis iónica, modulación de los neurotransmisores, diferenciación y migración de células gliales, e incluso unión de otras moléculas de la matriz al citoesqueleto de la neurona. Fuera ya de toda duda, se puede afirmar la existencia de una amplia matriz extracelular dentro del sistema nervioso y, por tanto, tener muy en cuenta la interacción neurona-matriz que, en ciertas localizaciones, podrá conferir diferencias funcionales sutiles a regiones específicas del SNC.[84]

En cambio, la **policaprolactona (PCL)** es un polímero sintético obtenido mediante la polimerización de un anillo abierto de ε-caprolactona. Su fórmula molecular es $(C_6H_{10}O_2)_n$ y tiene la característica de ser un polímero biocompatible,[85] semicristalino

y con un punto de fusión bajo de alrededor de 56°C, el cual variará en función de su peso molecular. Su degradación es lenta, lo cual es especialmente interesante cuando el implante debe permanecer un periodo prolongado hasta ser sustituido por tejido.[86,87]

El PCL es, a menudo, utilizado como aditivo para otros polímeros y para la fabricación de prototipos e implantes en biomedicina.[88,89] Su uso está aprobado por la FDA para aplicaciones biomédicas como polímero para liberación de principios activos,[88] ingeniería de tejidos, o suturas, etc.[90,91]

PLANTEAMIENTO DEL PROBLEMA

Dado que en una lesión iquémica, se produce una **pérdida de estructura** del parénquima cerebral por la muerte celular tanto de neuronas como de glia, el biomaterial tridimensional de ácido hialurónico actúa como un andamio apropiado para permitir la **re-organización** del tejido neural dañado con neuronas, glía y vasos sanguíneos.

Entre las características de la patología isquémica, se produce una gruesa **cicatriz glial** que aisla y sella la lesión del resto del parénquima, dificultando a nuevas células penentrar en ella y repararla. Por ello, la implantanción de un andamio poroso se realiza en la zona de penunbra, **atravesando** la cicatriz glial y estableciendo una comunicación parénquima - biomaterial - lesion.

El fallo bioenergético , el estrés oxidativo y la **reacción inflamatoria** tras el evento isquémico conducen a un **microambiente adverso**, incapacitándo a las células potencialmente recuperables retomar sus funciones. Es la administración de células madre mesenquimales de origen adiposo (ASCs) en la zona de penumbra, las que por su efecto **inmunmodulador y antiinflamatorio**, favorecen/mejoran el microambiente para la reparación del tejido.

A pesar de los procesos de reparación y repoblación mediante **neurogénesis** espontánea activados, éstos no son suficientes debido al escaso número de precursores que consiguen llegar al área diana. Las ASCs secretan **factores de crecimiento** que potencian la proliferación de células endógenas en SVZ.

El evento isquémico afecta a la unidad **neuro-vascular** y, en consecuencia, se produce una falta de neoformaciones de microvasos en el área de penumbra, limitando el acceso a nutrientes y oxígeno a células parcialmente dañadas. Las ASCs secretan **factores angiogénicos** que son clave en el crecimiento, mantenimiento y maduración de lso vasos sanguíneos.

En el momento en el que tractos de sustancia blanca se ven afectados, la regeneración axonal esta muy limitada, probablemente por la falta de factores quimio-atrayentes y/o la presencia de elementos inhibidores en el SNC. Por ello, la implantación de un andamio con un canal en su interior que comunique dos regiones distantes con células señalizadores (en nuestro caso, de la glía olfatoria envolvente) es capaz de atraer y guiar el crecimiento axonal.

HIPÓTESIS

La terapia celular combinada con biomateriales promueve los mecanismos de reparación endógena, favoreciendo la reducción del volumen isquémico y la reconexión de dos zonas distantes en el sistema nervioso central.

OBJETIVOS

Objetivo 1: Evaluar la **seguridad de las células madre mesenquimales** derivadas de tejido adiposo al administrarlas en el parénquima cerebral o vehiculizadas en un biomaterial de ácido hialurónico en un eventro isquémico.

Objetivo 2: Evaluar las **propiedades antiinflamatorias** de las células madre mesenquimales derivadas de tejido adiposo, así como **su efecto en sobre el proceso de neurogénesis** tras un accidente cerebrovascular isquémico.

Objetivo 3**:** Evaluar la **biocompatibilidad e integración** de un biomaterial tridimensional y poroso de ácido hialurónico implantado en el tejido cerebral, al igual que su **capacidad para ser colonizado** por células exógenas y endógenas, su superviviencia y maduración.

Objetivo 4**:** Evaluar el **efecto sinérgico** del biomaterial de ácido hialurónico precargado con células madre mesenquimales derivadas de tejido adiposo en una patología isquémica focal.

Objetivo 5**:** Evaluar la capacidad de un biomaterial de policaprolactona para actuar como puente, al implantarlo entre dos núcleos distantes del SNC, **para guiar el crecimiento axonal** entre ambos, y el efecto de la adición de células de la glia olfatoria envolvente de ratón para promoverlo.

OBJECTIVES

Objective 1: To evaluate the **safety of mesenchymal stem cells** derived from adipose tissue when administered in the brain parenchyma or transported in a hyaluronic acid biomaterial in an ischemic event.

Objective 2: To evaluate the **anti-inflammatory properties** of mesenchymal stem cells derived from adipose tissue, as well as their **effect on the process of neurogenesis** after an ischemic stroke.

Objective 3**:** To evaluate the **biocompatibility and integration** of a tridimensional and porous scaffold of hyaluronic acid implanted in brain tissue, as weel as its **capacity to be colonized** by exogenous and endogenous cells, its survival and maturation.

Objective 4**:** To evaluate the **synergistic effect** of the biomaterial of hyaluronic acid preloaded with mesenchymal stem cells derived from adipose tissue in a focal ischemic pathology.

Objective 5: To assess the ability of a polycaprolactone biomaterial to act as a bridge, by implanting it between two distant nuclei of the CNS, **to guide axonal growth** between the two, and the effect of the addition of mouse enveloping olfactory glia cells to promote it .

EVALUATION OF THE SAFETY AND EFFICACY OF THE THERAPEUTIC POTENTIAL OF ADIPOSE-DERIVED STEM CELLS INJECTED IN THE CEREBRAL ISCHEMIC PENUMBRA

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Evaluation of the Safety and Efficacy of the Therapeutic Potential of Adipose-Derived Stem Cells Injected in the Cerebral Ischemic Penumbra

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Introduction: Stroke represents an attractive target for cell therapy. Although different types of cells have been employed in animal models with variable results, the human adipose-derived stem cells (hASCs) have demonstrated favorable characteristics in the treatment of diseases with inflammatory substrate, but experience in their intracerebral administration is lacking. The purpose of this study is to evaluate the effect and safety of the intracerebral application of hASCs in a stroke model. *Methods:* A first group of Athymic Nude mice after stroke received a stereotactic injection of hASCs at a concentration of $4 \times 10^4/\mu L$ at the penumbra area, a second group without stroke received the same cell concentration, and a third group had only stroke and no cells. After 7, 15, and 30 days, the animals underwent fluorodeoxyglucose-positron emission tomography and magnetic resonance imaging; subsequently, they were sacrificed for histological evaluation (HuNu, GFAP, IBA-1, Ki67, DCX) of the penumbra area and ipsilateral subventricular zone (iSVZ). *Results:* The in vitro studies found no alterations in the molecular karyotype, clonogenic capacity, and expression of 62 kDa transcription factor and telomerase. Animals implanted with cells showed no adverse events. The implanted cells showed no evidence of proliferation or differentiation. However, there was an increase of capillaries, less astrocytes and microglia, and increased bromodeoxyuridine and

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doublecortin-positive cells in the iSVZ and in the vicinity of ischemic injury. *Conclusions:* These results suggest that hASCs in the implanted dose modulate inflammation, promote endogenous neurogenesis, and do not proliferate or migrate in the brain. These data confirm the safety of cell therapy with hASCs. **Key Words:** Stroke—ASC therapy—brain surgery—stem cells—animal model—intracerebral transplantation.

Introduction

Brain stroke is one of the most important conditions in health care, not only because of its high incidence, making it 1 of the 3 more frequent causes of mortality and the first one of disability in the western world, but also because of the high cost to the health-care system due to treatment, rehabilitation, and prevention of further episodes. Even if stroke treatment in the acute phase has improved ostensibly during the last decade, it is still a clinical situation provoking important neurological sequelae, which can lead to physical disability and dementia. Because of this, the search for alternative treatments devoted to the recovery of patients with stroke is a priority objective in clinical research.

The finding after experimental stroke of increased neurogenic activity, presence of endogenous neural stem cells at the cortex, and proliferation of nestin-positive cells both at the periphery and at the core of the ischemic zone[1,2] and of the survival of these cells at the ischemic area after permanent ischemia,[3-5] has boosted the idea that cell therapy may be effective for these patients. Different experimental studies support this possibility. The implant of stem cells induces a functional improvement in experimental stroke[6-10] and after the implant of immortalized pluripotent teratoma cells.[11] There are many possible mechanisms that could explain the reduction in infarct volume and neurological deficits found after ischemic stroke, such as replacement of neurons, neuroprotection, cell rescue via trophic support, promotion of endogenous neurogenesis, immunomodulation, or axonal plasticity, although the most probable one seems to be a local neuroprotective effect.[12] However, different experimental studies have shown that the generation of angiogenesis in the area close to the ischemia parallels the functional improvement in experimental stroke. The use of stem cells of mesenchymal origin has demonstrated to induce angiogenesis, and it could stimulate neurogenesis and the migration of neural cells to the ischemic area.[13]

It is because of this that the use of cells of mesenchymal origin has been proposed as a therapeutic possibility in stroke.[14] Among the mesenchymal stem cells, those derived from adipose tissue present a series of both biological and practical advantages making them interesting candidates for their therapeutic use. One of these advantages is that they induce angiogenesis through the liberation of cytokines as the vascular endothelial growth factor (VEGF).[15] Moreover, they present pathotropism especially to hypoxic areas. However, immunomodulatory effects have been described for mesenchymal cells both in vitro (they do not induce allogenic lymphocytic response and inhibit the lymphocytic response provoked by mitogens)[16,17] and in vivo (mesenchymal cells have controlled the graft versus host reaction associated to hematopoietic transplantations).[18]

Furthermore, these cells are widely available for their allogenic use, given the number of lipoaspirates done nowadays in plastic surgery, facilitating its donation. Different companies may provide cells from healthy donors, adequately processed and packed for their immediate use in humans. These characteristics make adipose tissue-derived stromal cells (ASCs) perfect candidates for stem cell therapy in stroke, and these cells have been used in various studies in experimental stroke.[19-29]

Some of the problems for the use of transplanted cells in the clinical treatment of stroke are the access pathway for the cells to the injured area and the understanding of the mechanisms of action. Although several studies have tested the use of cells delivered by the intravenous, intra-arterial, or intrathecal routes,[30-32] and the transplanted cells can migrate to the lesion area, their access to the area is difficult and their location in the penumbra zone is very sparse, which may foreshadow a beneficial effect. However, little is known about the mechanisms these cells could use to improve the conditions of the lesioned area when transplanted into the ischemic penumbra area. In addition, it is important to elucidate the safety aspects of this delivery, in terms of neurological, teratogenic, and inflammatory side effects.

In consequence, the objective of this study is to demonstrate the efficacy and safety of the direct brain injection of allogenic ASC in a stroke model administered into the ischemic penumbra area to proceed to translational clinical research.

Materials and Methods

Adipose Tissue Collection

Adipose tissue samples were obtained from donors during routine abdominoplasty following informed patient consent and according to the guidelines set by the corresponding ethics committee on biomedical research. The adipose tissue was transported in sterile bottles and was immediately

processed to obtain the ASC; this was done under Good Management Practices conditions (Histocell S.L.).

Isolation and Expansion of ASCs

The adipose tissue was rinsed with phosphate buffer solution (PBS) (Biochrom AG) containing 1% penicillin and streptomycin (Millipore, UK Ltd.) and incubated in a solution containing 1 mg/mL collagenase type II NB4 (SERVA Electrophoresis GmbH) for 1 hour at 37°C with vigorous shaking. The sample was disposed in 50 mL tubes (BD Biosciences) and centrifuged at 400 g for 10 minutes at room temperature. The top layer was removed and the pellet was resuspended in Dulbecco's Modified Eagle Medium-Glutamax (DMEM-Glutamax; Gibco, Life Technologies Corporation) and filtered through a 100-µm cell strainer followed by a 70-µm cell strainer (BD Biosciences). Cellular pellet was plated at a density of 60×104 mononuclear cells per cm^2 in 75-cm^2 culture flasks in DMEM-Glutamax supplemented with 10% fetal bovine serum (Biochrom AG) and 1% penicillin and streptomycin. Cell viability was determined using the trypan blue dye exclusion test in a hemacytometer.

In the following days, culture medium was replaced 3 consecutive days to remove residual nonadherent cells and tissue fragments. The cultures were maintained in a 5% CO_2 incubator at 37°C and the medium was changed 2 to 3 times per week. After reaching 80% confluence, the primary cultured cells (P0) were trypsinized using TrypLE Select (Gibco, Life Technologies Corporation). The cells were collected in 50-mL tubes and centrifuged at 400 g for 5 minutes at room temperature. The ASCs expansion was performed in 175-cm^2 culture flasks.

ASC were subsequently cultured for 5 more passages (P1, P2, P3, P4, and P5) in the same condition as P0. Total cell counts and cell viability was recorded for every passage. ASC immunophenotype, differentiation assay, colony forming units-fibroblast, 62 kDa transcription factor (c-Myc), and thelomerase assay are shown in Appendix S1.

In Vivo Studies

Animals and Surgical Procedures

Adult (7-week-old) male Athymic Nude-Foxn1NU/NU mice (from Envigo-Harlan Laboratories, Somerset, NJ) were housed in a temperature-controlled room with access to food and water ad libitum. They were caged individually under standard colony conditions in the animal facilities of the Hospital Clínico San Carlos. All procedures were performed under the standard criteria for ethical management and care of the animals in accordance with the EC Council Directive of November 24, 1986, and the procedures were approved by the local ethics committee.

$FeCl_3$ Thrombosis Model

Mice were anesthetized with 80-100 mg/kg ketamine and 10 mg/kg xylazine. A small craniotomy was performed, the dura was excised, and the middle cerebral artery exposed. Thrombus was induced by 1-minute topical application of a small strip of filter paper soaked with 20% $FeCl_3$ with the adventitial surface of the vessel.[33,34] In control group, .90% saline was used. $FeCl_3$ produces endothelial damage and denudation, which lead to thrombin-fibrin and platelet-dependent thrombus formation probably primarily via the collagen/GPVI axis.[35-37]

Cell Transplantation

After mice with stroke were randomly assigned to the treatment groups, stereotaxic surgery was performed under anesthesia 2 days after stroke for mice recovery from previous stroke surgery and regarding potential human transplantation. Mice were given 2 4.0-µl injections of ASCs or PBS (stroke groups) (4.4×10^4 cells/4 µl; or 4.0 µl of PBS) at the following coordinates: Antero Posterior:1.18; Medio Lateral:1.5; Dorso Ventral:2.5. A microinfusion pump was used to control the speed of delivery at .50 µl/min. The needle was left in situ for 10 minutes postinjection before being slowly removed. All the groups and a sham, which was not subjected to stroke and cell transplantation, were analyzed at different time points after cell transplantation (7, 15, and 30 days) (n = 5 per group and time point).

Behavioral Test

Behavioral analyses were performed 24, 48, and 96 hours after stroke according to the modified Neurological Severity Score proposed by Chen et al.[38] Neurological function was graded as 0-14 (normal score, 0; maximal deficit score, 14) by which motor and sensory function, balance impairment, and reflex abnormality were evaluated.

Magnetic Resonance Imaging (MRI)

Seven days after the surgery of cell implant, the mice were anesthetized with isoflurane 1.5%-2% for MRI. During the MRI procedure, the animals were kept at 37°C and an MRI compatible respiration sensor was used to control the animals. The MRI experiments consisted of three-dimensional (3D) T2 weighted image (T2WI), a series of diffusion-weighted imaging (DWI) to calculate the apparent diffusion coefficient (ADC) maps and a series of perfusion-weighted imaging to calculate cerebral blood flow maps. The perfusion experiments were carried out using arterial spin labeling techniques without contrast agent injection. All MRI experiments were performed on a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany) spectrometer operating a 4.7 T, equipped with an 11.2-cm actively shielded gradient system, capable of 200 mT/m gradient strength and 80 µs of rise time. A 7-cm birdcage

radiofrequency coil was used for transmission and reception. T2WI, DWI, and perfusion-weighted imaging image analysis was performed using ParaVision 3.0.1 (Bruker, Ettlingen, Germany). The ADC maps were calculated from the DWI series using the image sequence analysis tool of the ParaVision package. The cerebral blood flow maps were calculated from the arterial spin labeling images. This calculation was carried out using Matlab 7.3 (MathWorks, Inc., Natick, MA), the data were shown in % of brain volume affected.

^{18}-Fluorodeoxyglucose (^{18}-FDG) Positron Emission Tomography (PET) Imaging

At 30 days after cell implantation, the animals were subjected to analysis by PET imaging with ^{18}-FDG. ^{18}-FDG was used to assess glucose metabolism in the region of implantation, the mice before analysis were fasted for 8 hours and glycemia was controlled. Immediately following MRI acquisition, mice were injected intravenously with 18.5 MBq of ^{18}-FDG through the tail vein. After an uptake period of 60 minutes, the animals were anesthetized (isoflurane 1.5% in 100% oxygen) and placed on the bed with the head centered in the field of view in a spread prone position and scanned with the Albira small animal PET scanner (Oncovision, Valencia, Spain). PET images were coregistered with MRI images (T2-3D) obtained from each mouse. Briefly, the first step in the coregistration process was the automatic realignment of the volumetric 3D T2WI MRIs of the same mouse obtained at the different time points. Thereafter, 3D PET images were manually rotated and were shifted to match with their corresponding realigned MRIs. To this aim, the harderian glands from both PET and MRI scans were set as markers. In this process, a resolution adjustment was performed to extrapolate the 80°-80°-80 PET to 128°-128°-128 MRI matrix. The trilinear interpolation algorithm was applied, which uses all 8 enclosing pixel values. All these steps were carried out by using PMOD v 2.9 software (PMOD technologies, Zurich, Switzerland).

Immunohistochemistry and Morphometric Analysis

Four animals were sacrificed at different time points (7, 14, and 30 days after implant). Animals were perfused intracardially with 4% paraformaldehyde. Brains were removed, postfixed overnight, and cryoprotected in 15% sucrose overnight at 4°C. Subsequently, brains were frozen, and cryostat was sectioned coronally at 40 µm. Six serial series were prepared for volume analysis and immunohistochemical quantifications. Analyses were performed in a double blind.

Immunofluorescence

For bromodeoxyuridine (BrdU) immunostaining, sections were pretreated with 2N HCl (30 minutes at 37°C) and were then rinsed for 15 minutes in 0.1M borate buffer (pH 8.5). For CD31 immunostaining, sections were pretreated with 2% trypsin in .10% $CaCl_2$ (7 minutes at 37°C). Sections were rinsed in PBS and were incubated in blocking solution, followed by an overnight incubation at 4°C with the primary antibody: mouse anti-BrdU, 1:100 (DakoCytomation), mouse anti-human nuclei HuNu, 1:100 (Millipore), rabbit anti-GFAP 1:500 (DakoCytomation), rabbit anti-IBA1 1:500 (Abcam), mouse anti-CD31 1:100 (Abcam), or goat anti-doublecortin DCX, 1:200 (Santa Cruz Biotechnology). Subsequently, sections were washed and were incubated for 1 hour with the appropriate secondary antibody: mouse Alexa Fluor 555 or rabbit Alexa Fluor 488, goat Alexa Fluor 647, 1:500 (Invitrogen). The sections were mounted in FluorSave (Calbochem) with DRAQ5 (Abcam).

Neovascularization

Blood vessel density estimation was performed in the ischemic boundary region using an FV-1000 Olympus (Tokyo, Japan) motorized microscope. This region was delineated from the edge of the pan-necrotic cystic cavity approximately 300 µm into the adjacent cortex. Within these boundaries, 4 randomly fields at 10× magnification were analyzed. Blood vessel density was estimated as the percentage of CD31 + area in 10 sections per animal, spaced 240 µm apart, in the ipsilateral hemisphere (n = 4).

Inflammation

Microglia activation was evaluated by measuring their transformation shape.[36,37] Twenty-five microglial cells were randomly selected in a 300-µm wide area at the peri-infarct zone per section, in 6 coronal sections spaced 240 µm apart (150 microglial cells per animal) (n = 4 per group) 30 days after cell transplantation. The cell nucleus was placed in the center of a grid of 10 concentric 5 µm apart and the number of intersections between cytoplasmic processes and grid lines was registered using 100× oil immersion lens.

Glial Scar

GFAP expression in the border of infarct zone was quantified by measuring integrated optical density and area fraction with ImageJ software (National Institute of Mental Health, Bethesda, MA).

Subventricular Zone (SVZ) Proliferation

To evaluate the activation of SVZ proliferation, a single dose of BrdU (50 mg/kg) was injected 2 hours before the sacrifice of mice in each group (n = 4; sham, PBS, ASCs), which was done 30 days after transplantation. BrdU-positive cells in the SVZ were counted blindly in 40-µm coronal section per animal. Cells were counted under high power on an Olympus microscope. Results were

expressed as the average number of BrdU-positive cells per section.

DCX Cell Quantification

For counting the DCX-positive cells in SVZ, a fluorescent confocal microscope was set to 20× magnification, and all the immune positive cells have been counted in the particular regions. Counts are expressed as the average cell number per field ± standard error (SE) of means.

Endogenous Neuroblast Survival

The DCX/DAPI index was estimated in a series of 6 40-μm coronal sections after double labeling with DCX in the peri-infarct 30 days after cell transplantation.

Statistical Analysis

Statistical significance was assessed by 1-way analysis of variance followed by the Bonferroni post hoc test using GraphPad statistics software. A probability of $P < .05$ was adopted for statistical significance. Data are reported as mean ± SD.

Results

In Vitro Studies

Doubling Time and Generations Number

ASCs showed plastic-adherent properties in culture. Cells showed normal spindle-like fibroblastic morphology and cell morphology did not differ from the first to subsequent cultures (Fig 1). Phase-contrast microscopy pictures showed an optimal cell growth in the culture flask surface. After reaching subconfluence, cells were detached from the culture surface to calculate the doubling time and number of generations. In all cases, doubling time of ASC was less than 3.2 days and the number of generations was less than 3.5 in each passage with a cumulative total generations of 11.3 (Table 1).

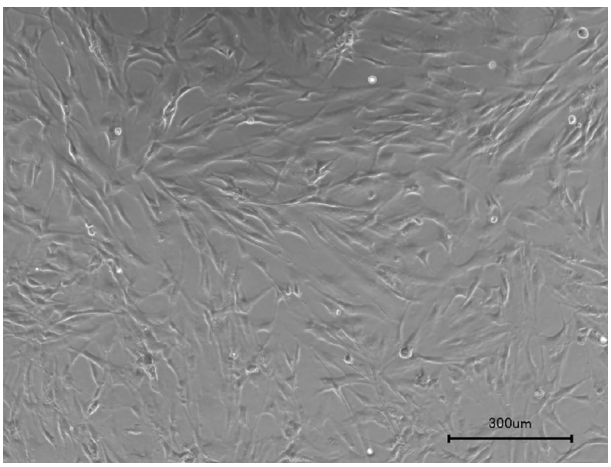

Figure 1. *Phase-contrast microscopy pictures of cultured ASCs, showing normal spindle-like fibroblastic morphology and an optimal cell growth. Abbreviation: ASC, adipose tissue-derived stromal cells.*

Table 1. *Doubling time and generation number of ASC*

Passage	Doubling time (g;days)	Generations number (n)
P1-P2	2,7	2,3
P2-P3	3,2	2,8
P3-P4	2,0	3,5
P4-P5	2,6	2,7
Cumulative		11,3

In Vivo Studies

In none of the animals in which ASCs were injected, clinical events showing adverse effects were observed (convulsions, epileptic seizures, aggressivity, hyperexcitability, or depressed activity). The neurological evaluation[38] showed deficits only in the animals subjected to brain ischemia at 24 hours after ischemia induction. No other deficits were found 72 hours later neither in experimental animals nor in the sham animals. The observed data are shown in Table 2.

The macroscopic analysis of the lesion showed no evidence of infection, inflammation, or necrosis. Animals receiving ASCs showed a less profuse surgical scar. In the analysis of the organs of the animals where ASCs were implanted, no macroscopic or microscopic tumoral cell formations were found.

Imaging Studies

At 30 days after implantation, there was no evidence of tumor formation in the MRI performed to the animals receiving ASC injection. Furthermore, the analysis of the images taken of animals implanted with ASC showed less ischemic area than controls (Fig 2A). The data show differences between the stroke and stroke group with ASC, with 8.65% (+2.543 SE) of lesion volume in animals with stroke and 4.13% (+1.57 SE) in animals where cells were implanted after the stroke, the data being statistically significant (Fig 2B). ^{18}-FDG PET studies showed no cell masses in the brain with increased glucose uptake (data not shown).

Angiogenesis

In the group of animals receiving ASCs, an increment of vessels was observed in the vicinity of the lesion or at the implant site. This phenomenon was also seen in the ischemia control group, showing a discrete increase, but the increment in the ASC group was higher and statistically significant compared to the ischemic or sham control groups (sham: 2.49% ± .19, 2.47% ± .27, 2.38% ± .19;

Table 2. *Neurological Severity Scores (NSS)**

		Group					
		Sham		Stroke		Stroke + ASC	
	Maximum points	1 h	24 h	1 h	24 h	1 h	24 h
Motor tests							
Raising rat by the tail	3	1,66	0,00	2,33	1,16	2,16	0,50
Placing rat on the floor	3	0,16	0,00	1,50	0,83	1,16	0,16
Sensory test	2	0,00	0,00	1,00	0,00	1,00	0,00
Beam balance test	6	0,00	0,00	2,33	0,66	2,16	0,50
Reflex's absent and abnormal movements	4	0,46	0,16	1,83	0,00	1,66	0,00
Maximum points		2,28	0,16	8,99	2,65	8,14	1,16

For the inability to perform the tasks or lack of a tested reflex, 1 point is awarded; 13 to 18 indicate severe injury; 7 to 12 indicate moderate injury; and 1 to 6 indicate mild injury.

The data are the observed average.

*From Chen et al, 2001.[38]

stroke: 3.93% ± .36, 2.99% ± .28, 2.76% ± .23; and stroke + ASC: 6.15% ± .61, 4.71% ± .44, 3.93% ± .36 at 7, 15, and 30 days respectively), $P < .05$ (Fig 3).

Analysis of Microglia and Glial Scar

Evaluation of the inflammatory response using IBA-1 expression analysis (by immunofluorescence and quantitative ring analysis) in microglial cells 7 days after implantation showed that animals suffering ischemia presented transformation of microglial cells from a quiescent branched form (Fig 4A and D) to a phagocytic amoeboid morphology (Fig 4B-E). These changes were more evident in the ischemic control group (Fig 4B and C), ($P < .05$) (sham: 33.30 ± 1.09; stroke: 10.75 ± .48; and stroke + ASC: 15.75 ± .69, data in number of intersections/rings), ($P < .05$) (Fig 4F).

Glial scar analysis by immunofluorescence analysis of GFAP expression at the ischemic border at 7 days showed that animals in the ischemic control group presented an

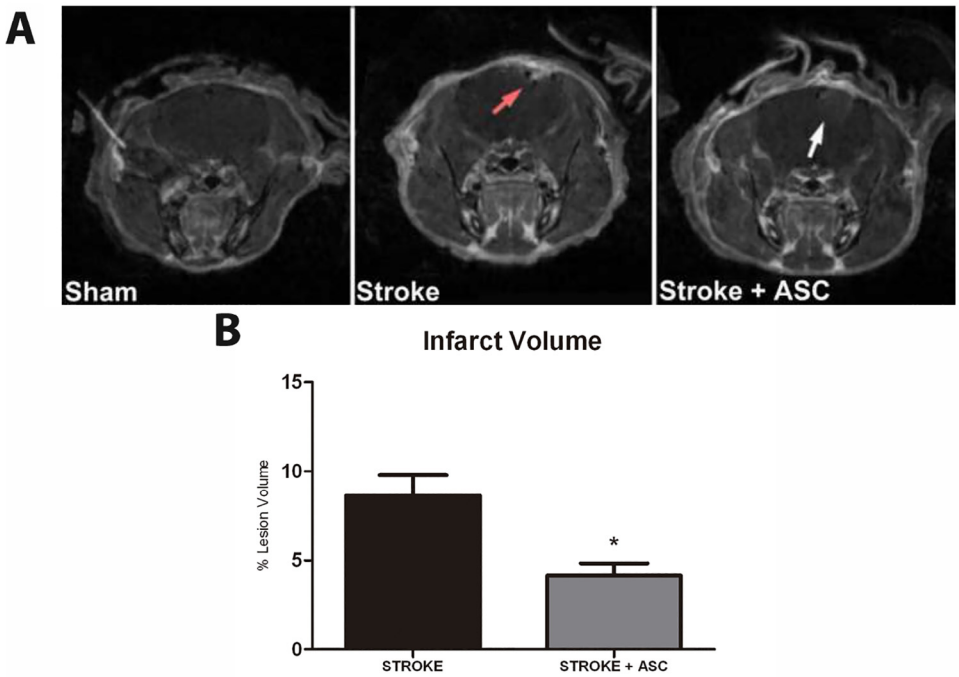

Figure 2. *(A) MRI images of the animal's brain at 30 days after implantation. There was no evidence of tumor formation in the MRI performed to the animals receiving injection of ASCs. Furthermore, the analysis of the images taken of animals implanted with ASCs showed a smaller ischemic area than controls (arrows). (B) The graph shows the percentage of stroke volume at 30 days, observing a significant lower volume in the ASCs group. Abbreviations: ASC, adipose tissue-derived stromal cells; MRI, magnetic resonance imaging.*

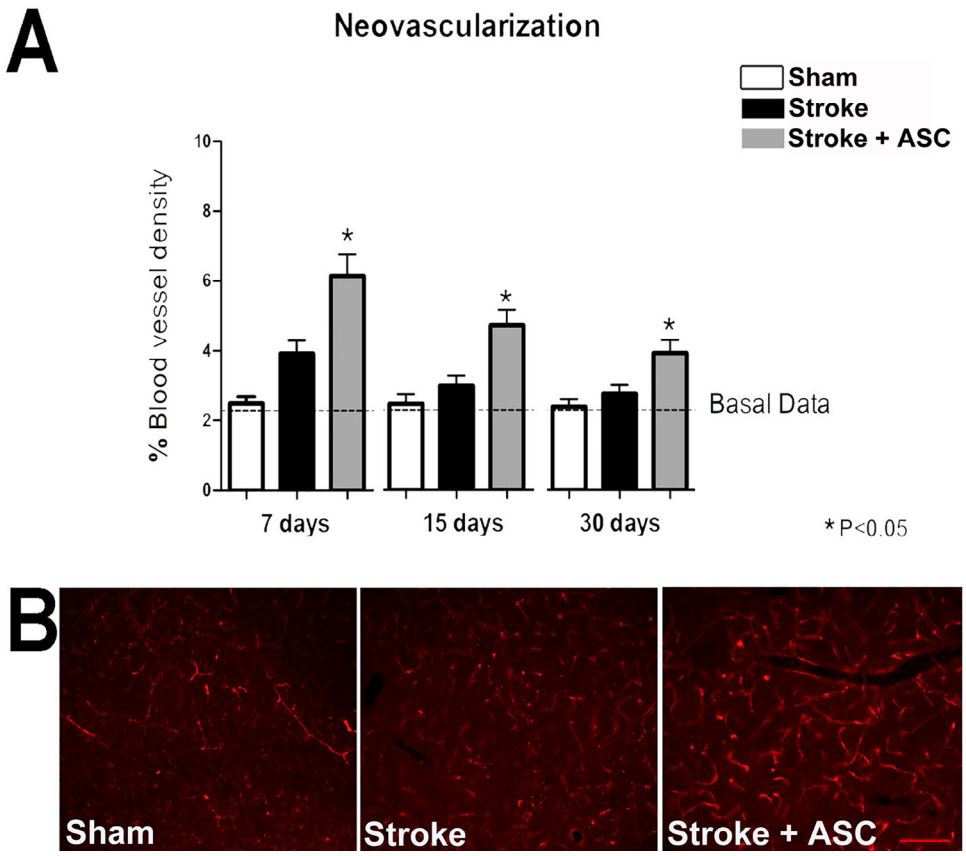

Figure 3. *Neovascularization at the stroke zone after ischemia. (A) The graph shows the positive effect of ASC injected after stroke at the 3 analysis time points. (B) Confocal microscopy images at 30 days after implantation, showing an increase of the capillaries at the area of implant. Scale bar: 50 μm. Abbreviation: ASC, adipose tissue-derived stromal cells.*

increase of GFAP expression, while animals receiving ASC after stroke showed less GFAP expression, without reaching the basal or the sham group values (sham: .53 ± .03; stroke: 1.78 ± .10; and stroke + ASC: .935 ± .08, data expressed in optical density, basal data: .308 ± .02), (*$P < .05$, **$P < .01$) (Fig 4F-I).

Cell Proliferation and Cellular Implant

When we evaluated if the ASCs (HuNu positive) colocalized the expression of BrdU, there was no evidence of this double marking (Fig 5). When analyzing if the implant procedure or the ischemia induces an increase of cellular proliferation, we found that the SVZ ipsilateral to the ischemia showed a considerable increase of BdrU-positive cells when compared to the sham group (Fig 6A-C). Animals receiving ASC showed even a further increase of proliferation at the SVZ (sham: 52.63 ± 3.43; stroke: 69.63 ± 2.41; and stroke + ASC: 84.75 ± 3.31; data expressed as BdrU + cells per section), (*$P < .05$, **$P < .01$) (Fig 6A-D).

Activation of Neurogenesis after Lesion

To determine if the implant of ASC promotes the generation of neuroblasts at the SVZ, and the migration and survival of endogenous cells in the brains of animals after stroke, we performed an immunostaining against DCX (marker of young neurons).

It was evident that any stimulus in the brain induced an increase of DCX expression at the SVZ, but animals with stroke plus ASC showed a marked activation at the ipsilateral SVZ (sham: .54 ± .06; stroke: 1.01 ± .05; and stroke + ASC: 1.691 ± .10, data expressed in optical densities, basal data: .51 ± .03), *$P < .05$, **$P < .01$ (Fig 7B-D).

Furthermore, an increase of DCX-positive cells was found at the peripheral boundary of the stroke zone in animals treated with ASC when compared with animals with only stroke. The estimation of the DCX/DAPI index confirmed the increase of DCX-positive cells (sham: .029 ± .004; stroke: .061 ± .009; and stroke + ASC: .110 ± .021), *$P < .05$, (Fig 8).

Discussion

Although different meta-analyses have examined the benefit of cell transplantation in experimental stroke,[39] the question of the route for the access of the cells to the damaged area is still in question. While those who consider the mechanism of action of the cells may be based on neuroprotective effects that may act remotely defend

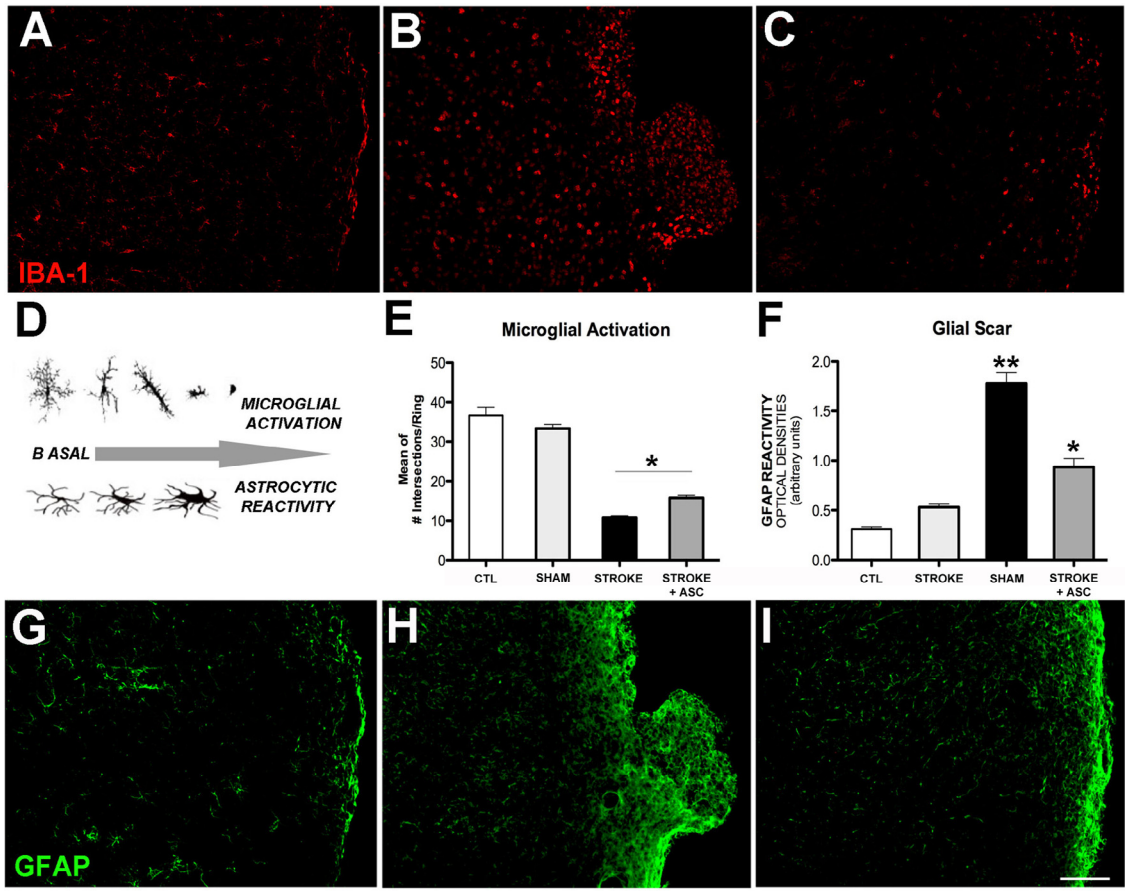

Figure 4. *Analysis of microglia and glial scar. (A-C) Evaluation of the inflammatory response using IBA-1 expression analysis of microglial cells 7 days after implantation. Ischemic animals showed increased phagocytic activation compared to animals with implanted ASC, demonstrating a modulating effect on inflammation of the ASC. (D) Morphological scheme of the microglial and astrocytic response to injury. (E) Analysis of the microglial activation after brain ischemia, showing a modulatory effect of ASC. (F) The graph shows the effect of ASC on the reduction of the glial scar produced after ischemia. (G-I) Confocal images of the glial scar (GFAP expression at the ischemic border at 7 days), the animals receiving ASC after stroke showing less GFAP expression. Scale bar A-C, G-I: 100 μm. Abbreviations: ASC, adipose tissue-derived stromal cells.*

a systemic access, other authors that consider that the role of angiogenesis and neurogenesis is important prefer to implant the cells at the site of injury. In this sense, high accumulation of cells to the lungs and other organs is a significant problem after intravenous cell transplantation, and intra-arterial administration does not seem to induce angiogenesis in the damaged area.[40,41] It is not clear that the transplanted cells remain a long time in the damaged area, not more than 2 hours in the intravenous[42] and similarly for the intra-arterial route.[43] Although the direct access of the transplanted cells to the damaged area may seem a reasonable delivery option, very few studies have addressed this route. However, one of the issues that might arise as a result of the direct implant into the tissue is the potential risk of tumoral transformation.[44-46] Our study has explored the possibility of introducing these cells directly into the ischemic penumbra area of damaged tissue and our results show that ASC "in vitro" behave in a stable way and do not express tumoral markers after several passages, and that the implantation of these cells "in vivo" does not appear to increase the risk of neoplastic transformation.

The in vitro behavior of these cells corresponded, as expected, to a stable one: in plastic-adherent properties in culture, spindle-like fibroblastic morphology, stability through all culture passages, and a normal doubling time[47] up to 5 passages (data shown in Appendix S1). ASCs were able to differentiate into adipocytes, chondroblasts, and osteoblasts. CFU-assay was performed to evaluate the clonal expansion capacity of ASC, demonstrating that ASC cultures contained a subpopulation of cells capable to generate new fibroblast-like colonies from single cells, with a 51.7% of clonal efficiency. The enzyme telomerase and the proto-oncogene c-Myc are typical markers of alterations in the cell cycle that are overexpressed in tumoral cells. ASC did not show alterations in the in vitro expression of these markers, with similar values as those reported for the basal values in cells with a stable proliferating capability[48] and in none of the in vitro passages or in

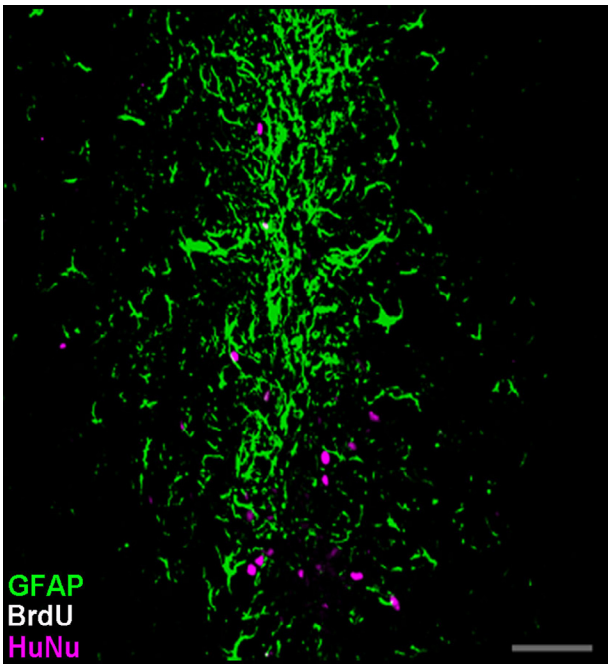

Figure 5. *ASC and proliferation. Confocal microscope showing expression of HuNu and BrdU. None of the HuNu marked cells express BrdU, indicating a lack of proliferation of the implanted cells after BrdU injection. Scale bar: 50 μm. Abbreviations: ASC, adipose tissue-derived stromal cells; BrdU, bromodeoxyuridine.*

vivo, there was expression of c-Myc or telomerase, while they were overexpressed in the positive controls (SF767 cell line).[49]

Our study shows that in vivo, these cells did not produce undesired effects; on the contrary, they promoted angiogenesis, induced proliferation, and migration of neuroblasts to the ischemic zone and contributed to a less extensive ischemic lesion. No adverse neurological effects were observed in the implanted animals, except a mild deficit in all animals subjected to the stroke model (both those receiving ASC and controls) at 24 hours, with a full subsequent recovery. In addition, no signs of tumor formation were observed in any animals receiving ASC neither histologically nor in the imaging studies, nor any sign of infection or necrosis, with a less prominent scar, and less ischemic area in animals receiving ASC. An increment of vessels was observed in the vicinity of the lesion or at the implant site in the group of animals receiving ASC. It is known that after ischemic lesions or after rupture of the blood brain barrier, there is an increment of vascular growth promoting molecules, such as CD31, VEGF, endothelins, and proteoglycans among others.[50] Thus, angiogenesis was also seen in the ischemia control group, but the increment was significantly higher in the ASC group. Some of our objectives were to evaluate if the implanted cells proliferate and if the implant of these cells produces a proliferative response at the SVZ, described as a neurogenic niche in the adult brain. BrdU is a thymidine analog; when it is administered before the sacrifice of an animal, it permits to know the existing proliferation at a given moment of an experimental condition. When we evaluated if the ADC (HuNu positive) colocalized the expression of BrdU, there was no evidence of this double marking, indicating that ADC do not proliferate at the implant site. This has been already described in biopsies of patients receiving ASC, not showing any tumoral growth.[51]

Arvidsson et al showed an increase of cellular proliferation at the SVZ ipsilateral to the stroke.[2] To determine if the implant of ASC promotes the generation of neuroblasts at the SVZ, and the migration and survival of endogenous cells in the brains of animals after stroke, we performed an immunostaining against HuNu (transplanted cells) and DCX (marker of young neurons). It was evident that any stimulus in the brain induced an increase of DCX expression at the SVZ, but it is interesting that animals with stroke plus ASC showed a much stronger activation at the ipsilateral SVZ. Moreover, an increase of DCX-positive cells was found at the peripheral boundary of the stroke zone in animals treated with ASC when compared with animals with only stroke. It seems that ASC induces a greater survival of neuroblasts at the peripheral boundary of the stroke zone, and this indicates that possibly the transplant ASC has additional neurogenic and neuroprotective effects.

The exact mechanism of the beneficial effects of mesenchymal stem cells is unknown. While direct differentiation of these cells is possible but unlikely,[52] neuroprotective effects at the penumbra zone have been quoted as the main mechanism of the behavioral improvement associated to the administration of these cells.[53] However, other factors, such as proliferation and migration of neuroblasts and angiogenesis, may contribute to neuroprotection and even to neural tissue regeneration. In any case, the stem cells seem more resistant to ischemia than more differentiated cells.[54] Thus, stimulating the migration of neural stem cells can be a beneficial mechanism to improve the stroke condition.

Although several mesenchymal stem cells or other types of cells have been used for the treatment of stroke, both in animals[3] and in clinical trials,[16] ASCs present a series of both biological and practical advantages making them interesting candidates for their therapeutic use. As noted previously, they induce angiogenesis through the liberation of cytokines such as VEGF, and they present pathotropism, especially to hypoxic areas. Both effects would be important in the therapy of ischemic processes.[17] Moreover, they have immunomodulatory effects both in vitro[55] and in vivo.[56] Furthermore, these cells are widely available for their allogenic use, as they can be obtained from lipoaspirates in plastic surgery, facilitating its donation. Different companies process and pack those cells from healthy donors, making them available for their immediate use in humans. Thus, ASCs are good candidates for their use in stroke therapy.

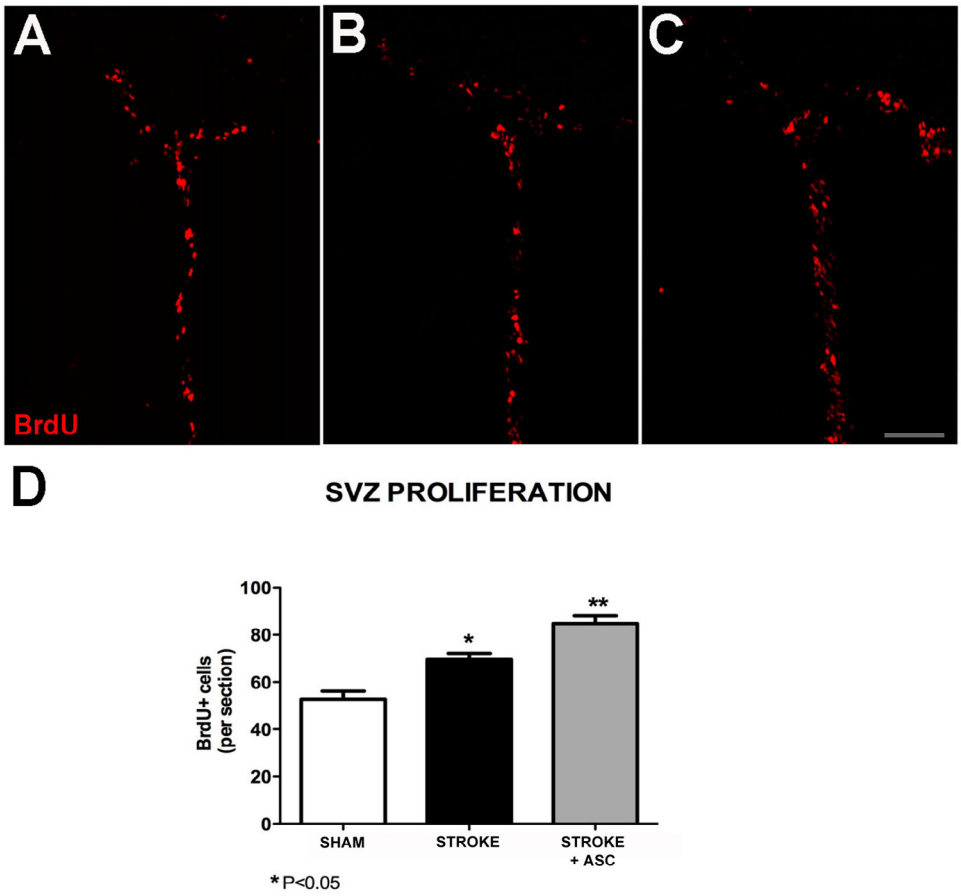

Figure 6. *Effect of ASC injection on the SVZ after stroke on cell proliferation at the SVZ. (A-C) confocal microscopy showing BrdU expression at the SVZ. This expression is increased in animals with stroke, and it is even more increased in ASC-injected animals. (D) Graphic quantitation of cell proliferation at the SVZ. Scale bar: 100 μm. Abbreviations: ASC, adipose tissue-derived stromal cells; BrdU, bromodeoxyuridine; SVZ, subventricular zone.*

The choice of factors such as the moment and the route of administration can be critical for the clinical outcome. Of the clinical trials published until the last years, one[15] uses the intravenous route and includes acute stroke patients, with a first intravenous injection at 4-6 weeks and a second one at 7-9 weeks. The rest of the studies use the direct brain implant of cells[57,58] and perform the interventions between 3 months and 6 years after stroke. In this context, it can be important to reduce the time inverted from the extraction to the delivery of viable cells. For this, the use of allogenic cells available commercially is a proper option, given that culture of autologous cells can be uncertain (some cultures may not thrive) and the needed quality controls may delay the time between extraction and delivery.

Our data contribute to the discussion on the route of administration that has been controversial. Some experimental studies suggest that several stem cells induce recovery when administered both through the intrathecal and intravenous routes. However, clinical data suggest that the greatest benefit is obtained when they are directly injected into the brain parenchyma, in the vicinity of the healthy zone around the penumbra area. Possibly this is related to the size of these cells, which crosses, with difficulty, the blood brain barrier, and with their filtration and elimination from the circulation at the first step after the intravenous delivery. However, the expected effect is through the promotion of angiogenesis, and this has only sense if they are locally delivered at the ischemic areas. The increase of angiogenesis at other healthy sites could provoke side effects, such as brain steal. Other sought-after effects are the anti-inflammatory and the neuroprotective one, and this is probably more effective if a higher local concentration is achieved through its intracerebral local delivery.[59-61]

We have demonstrated that ASC induces no side effects per se, but the lack of stable neurologic side effects, even in animals subjected to stroke without ASC, impedes to study the possible beneficial clinical effect of these cells. This kind of study should be performed in a more sensitive stroke model in rodents or in other species.

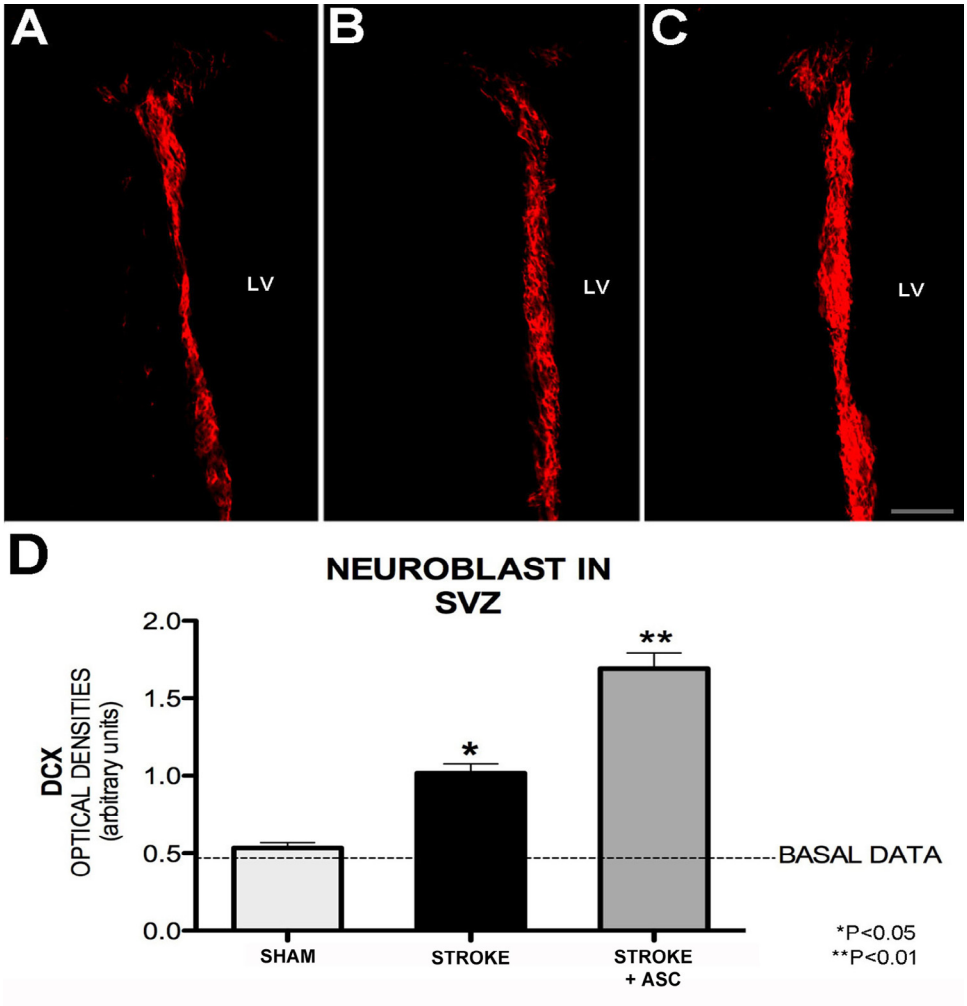

Figure 7. *Effect of ASC injection on the SVZ after stroke on neuroblast proliferation at the SVZ. (A-C) confocal microscopy showing doublecortin (DCX) expression at the SVZ. This expression is increased in animals with stroke, and it is even more increased in ASC-injected animals. (D) Graphic quantitation of cell proliferation at the SVZ. Scale bar: 100 μm.*

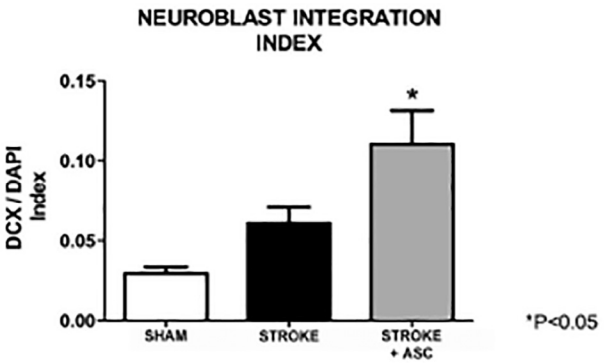

Figure 8. *Integration of DCX at the periphery of the ischemic lesion area. The graph shows a significant increase of DCX cells at the periphery of the lesion in animals receiving ASC. Abbreviations: ASC, adipose tissue-derived stromal cells; SVZ, subventricular zone.*

In conclusion, this study shows that ASC implanted via the intracerebral route in a model of mice with stroke is safe. Cells implanted at the penumbra zone increase angiogenesis and cell proliferation and migration from the SVZ. This may lead to the use of direct cell implantation as a more effective delivery method for stem cell therapy of stroke. Our work opens the possibility of using ASC as an alternative cell source in the local treatment of stroke in humans.

Appendix: Supplementary Material

Supplementary data to this article can be found online at doi:10.1016/j.jstrokecerebrovasdis.2018.05.001.

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BIOHYBRIDS OF SCAFFOLDING HYALURONIC ACID BIOMATERIALS PLUS ADIPOSE STEM CELLS HOME LOCAL NEURAL STEM AND ENDOTHELIAL CELLS: IMPLICATIONS FOR RECONSTRUCTION OF BRAIN LESIONS AFTER STROKE

JOURNAL OF BIOMEDICAL MATERIALS RESEARCH PART B

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Biohybrids of scaffolding hyaluronic acid biomaterials plus adipose stem cells home local neural stem and endothelial cells: Implications for reconstruction of brain lesions after stroke

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Abstract: Endogenous neurogenesis in stroke is insufficient to replace the lost brain tissue, largely due to the lack of a proper biological structure to let new cells dwell in the damaged area. We hypothesized that scaffolds made of hyaluronic acid (HA) biomaterials (BM) could provide a suitable environment to home not only new neurons, but also vessels, glia and neurofilaments. Further, the addition of exogenous cells, such as adipose stem cells (ASC) could increase this effect. Athymic mice were randomly assigned to a one of four group: stroke alone, stroke and implantation of BM, stroke and implantation of BM with ASC, and sham operated animals. Stroke model consisted of middle cerebral artery thrombosis with $FeCl_3$. After 30 days, animals underwent magnetic resonance imaging (MRI) and were sacrificed. Proliferation and neurogenesis increased at the subventricular zone ipsilateral to the ventricle and neuroblasts, glial, and endothelial cells forming capillaries were seen inside the BM. Those effects increased when ASC were added, while there was less inflammatory reaction. Three-dimensional scaffolds made of HA are able to home newly formed neurons, glia, and endothelial cells permitting the growth neurofilaments inside them. The addition of ASC increase these effects and decrease the inflammatory reaction to the implant. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res B Part B: 00B: 000–000, 2018.

Key Words: biomaterials, hyaluronic acid, cell therapy, adipose stem cells, stroke

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INTRODUCTION

In spite of the extensive pharmacological and neurorehabilitation efforts made to recover the neurological deficits, stroke is still the main cause of disability in the occidental world. Ischemic stroke produces an area of necrosis (core zone) surrounded by a partially damaged zone (area of penumbra).[1] Most of the therapeutic efforts up to now have been directed to reduce the extent and to improve the functional recovery of the penumbra zone.[2] However, there is not a reliable way to repair the lost tissue.[3]

After an experimental stroke, there is an increase of the neurogenesis at the subventricular zone (SVZ), a main adult

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neurogenic niche, especially at the ipsilateral hemisphere, and new neurons and supporting cells migrate massively to the area of infarction.[4] However, most of these cells disappear from the lesion by the 5th week.[5] We hypothesize that the main reason for this is the lack of a proper substrate where the cells can survive, reconnect, and recover their function due to the generation of an adverse environment after the stroke[6,7] and/or to the absence of a material scaffold upon which new neurons and supporting structures such as glia and vessels can grow and organize.

The use of three-dimensional (3D) biomaterials (BM) could provide a proper scaffold for these newly formed cells, permitting the organization of neural tissue with neurons, glial cells and vessels, guiding the neurite prolongations and providing a more appropriate environment for neural regeneration.[8–12] Among the multiple available biocompatible materials, we chose hyaluronic acid (HA) because this is an already used BM in other clinical applications and it is simple to make and manipulate.[13,14]

Furthermore, mesenchymal stem cells have been shown to home endogenous stem cells close to the infarcted area, as well as to increase neovascularization at the same site and proliferation at the SVZ, and also to diminish the inflammatory reaction at the lesion site.[15–18] We hypothesized that the addition of mesenchymal stem cells to the BM would increase their capacity to home endogenous stem cells, and possibly to facilitate their entry into the BM by inhibiting the scar reaction around the scaffold.[19,20] Among the mesenchymal cells available, we have chosen adipose stem cells (ASCs) because of practical reasons: they are easily handled, they are widely available from lipoaspirate donation for allogenic use and our group has already shown their potential benefits for clinical application in stroke.[21–23]

The objectives of this study were to determine if 3D scaffolds of hyaluronic acid (HA) implanted at the penumbra zone of a vascular stroke lesion in rats were able to home local neural and endothelial cells and neurites, and also to compare these homing capacities between BM co-grafted with adult human ASC and BM without them, as well as to study the host's reaction to the implant in both cases, both in terms of glial scar formation and neurogenetic production. Also, if this represents a viable and safe method in order to transfer this to the clinical practice.

MATERIALS AND METHODS

Preparation of HA porous scaffolds

HA porous scaffolds were made as described Rodríguez-Pérez et al.[24] Briefly, HA sodium salt from Streptococcus equii (1.5–1.8 MDa; Sigma-Aldrich) was dissolved overnight to a 5% (w/v) in 0.2 M sodium hydroxide (NaOH, Scharlab). Then, divinyl sulfone (DVS, Sigma-Aldrich) was added as a crosslinker in a 9:10 DVS:HA monomeric units molar ratio. Once homogenized for 10 s more, the solution was vaccuum-injected into molds made from sintered beads of poly (ethyl methacrylate; PEMA, Elvacite® 2043, Lucite International, Inc.). These beads had a diameter of between 180 and 250 μm. The crosslinking reaction of HA with DVS was left to complete for an hour and a half.

After that, PEMA beads template was removed by leaching reflux in a Soxhlet extraction apparatus using boiling ethanol as solvent for 48 h. Afterwards, HA scaffolds were immersed in distilled water for 3 h, frozen at −20°C for another 2 h and then lyophilized. All scaffolds were cut into cylinders with 0.8 mm diameter and 8 mm of length. Other tests were carried out to warrant the absence of pyrogens or endotoxins, to guarantee biomaterial's innocuity.

Analysis of 3D structure of biomaterials

The HA scaffolds, previously sterilized by chemical means, were analyzed by scanning electron microscopy to analyze the 3D configuration of the scaffold, ensuring that pores are between 300 and 150 um in diameter were present, so that ASC can easily enter the BM. For this purpose, they were processed in a critical dot dryer, followed by gold–palladium shading to provide electroconductivity to the sample and analyzed in a scanning electron microscope JEOL JSM 5410 with a voltage of 10 KV at different magnitudes (Figure 1).

Adipose stem cells

Adipose tissue samples were obtained from donors during routine abdominoplasty following informed patient consent and according to the guidelines set by the corresponding

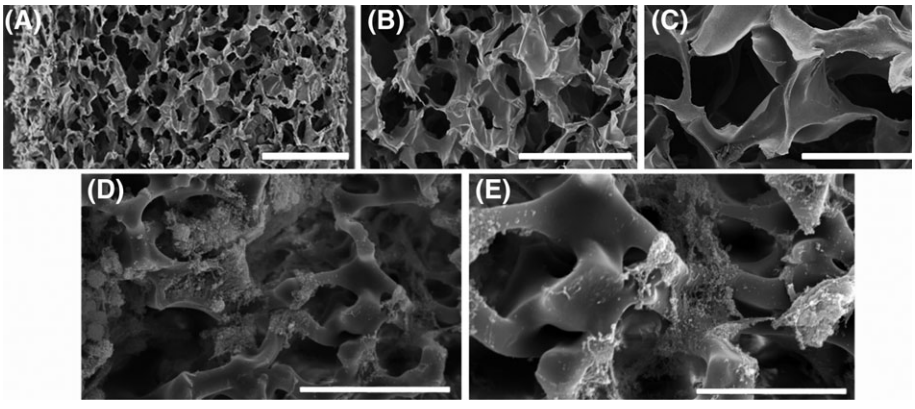

FIGURE 1. Scanning electronic micrographs showing porous scaffolds made of hyaluronic acid at different magnifications (A–D). A–C: shows images of the biomaterial and the characteristics of the scaffolding and D–E: images of ASC cells colonizing the scaffold. Scale Bar A: 500 μm, B: 300 μm, C: 150 μm, D: 80 μm, and E: 30 μm.

Ethics Committee on Biomedical Research. The adipose tissue was transported in sterile bottles and immediately processed to obtain the ASC and process under GMP conditions (Histocell, S.L., Bilbao, Spain). Phenotype characteristics, clonic capacity and stem cell differentiation biomarkers as described by Gomez-Pinedo et al.[23]

The ASC were maintained until phase 7 in culture on the material to perform studies of telomerase and C-myc expression, to guarantee the stability of the cultured cells on the biomaterial. Before use, we proceeded to carry out the immunophenotype of the ASC cells to be considered mesenchymal stem cells and useful for our purpose.

Scaffold preloading

ASC were used in the fourth cell phase. Cells were resuspended in culture medium at a concentration of $5x10^4/4$ µL. This concentration was inoculated inside the HA biomaterial (dimensions for the preclinical study of 1 x 1 mm) with the help of a Hamilton® microsyringe. The biomaterials inoculated with ASC were maintained in 24-well plates with the culture medium composed of DMEM-Glutamax + Antibiotic / 10% FBS and incubated at 37°C/5% CO_2 for 72 h, for subsequent implant in the model of athymic nude mice [Figure 1(D–E)].

$FeCl_3$ thrombosis model

Nude-Foxn1^{NU}/^{nu} (dEnvigo -Harlan Laboratories) adult male mice were used for the experiments. At 7 weeks of age were housed in a temperature-controlled room with access to food and water ad libitum. They were kept in individual cages and under standard conditions in the animal facilities of the Hospital Clínico San Carlos. All procedures were carried out under animal welfare regulations in accordance with EC Council Directive November 24, 1986 and approved by the local ethics committee.

Mice were anesthetized with 80–100 mg/kg ketamine and 10 mg/kg xylazine. A small craniotomy was performed to access the middle cerebral artery. For the formation of the ischemia model, a $FeCl_3$ solution (20%) was applied to the surface of the artery for 1 min. In the control group (sham) 0.9% of saline solution was used.[15,16]

Experimental and control groups

Four groups were defined: Stroke (mice subjected to the stroke model), Stroke+BM (mice subjected to the stroke model to whom HA biomaterials alone were implanted at the core zone), Stroke+BM+ASC (mice subjected to the stroke model to whom HA biomaterials co-grafted with ASC were implanted at the core zone) and sham (animals undergoing the operation for the stroke model except the application of the thrombotic $FeCl_3$ solution). Six animals were assigned randomly to each group.

Seven days after the ischemic procedure, animals belonging to groups Stroke+BM and Stroke+BM+ASC were again anesthetized and the wound reopened. A burr hole was performed at the cranial vault just medial to the temporal crest

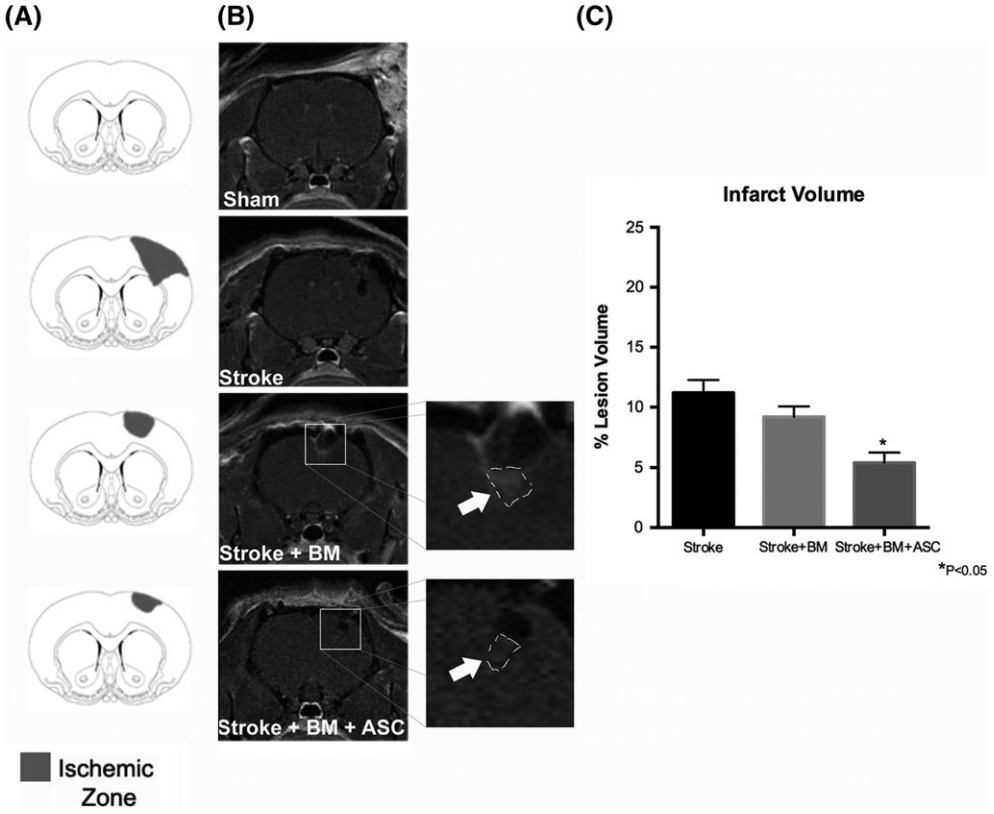

FIGURE 2. Magnetic resonance imaging demonstrating the infarcted area in the control group (only stroke) and the implanted groups. In the latter, the biomaterial is fully integrated, and it is associated with less cavitation.

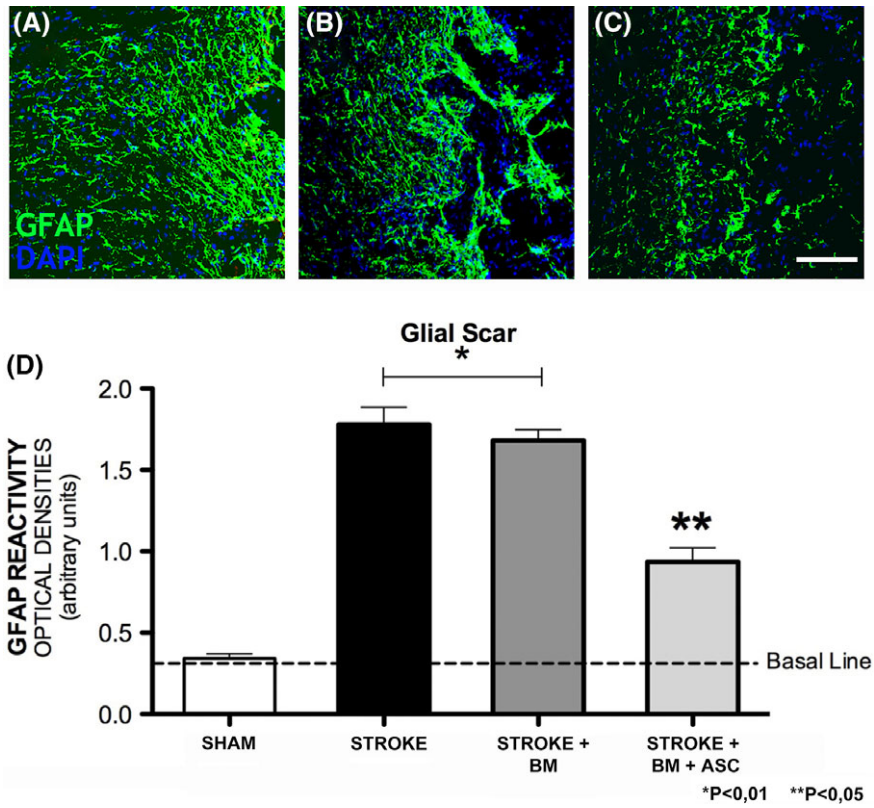

FIGURE 3. Analysis of the glial scar. Representative immunohistochemical image showing glial fibrillary acidic protein (GFAP), (A) stroke; (B) stroke +BM; (C) stroke+BM+ASC; (D) The graph shows that in the group of animals with stroke, in which ASC were administered in biomaterials, they showed less expression of the GFAP, in the other two groups (stroke and stroke+BM) the expression of GFAP was significantly higher (**$p < 0.05$ and *$p < 0.01$) Scale Bar: 100 μm.

at the same coronal plane at which the occluded MCA branch was located. A cannula was inserted to implant the biomaterials (with or without ASC) at a depth of 13 mm from the dural plane. (Supporting Information Figure 2).

Magnetic resonance imaging

The day of the sacrifice, the mice were anesthetized with isoflurane 1.5–2% to obtain a brain MRI. All the MRI experiments were performed on a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany) spectrometer operating a 4.7 T, equipped with a 11.2 cm actively shielded gradient system, capable of 200 mT/m gradient strength and 80 μs of rise time. During the MRI procedure, the animals were kept at 37°C and a MR compatible respiration sensor was used to control the animals. The MRI experiments consisted of three-dimensional T2WI, a series of DWI to calculate the ADC maps and a series of PWI to calculate CBF maps. A 7-cm birdcage radiofrequency coil was used for transmission and reception. Image analysis was performed using ParaVision 3.0.1 (Bruker, Ettlingen, Germany). This calculation was carried out using MATLAB 7.3 (MathWorks, Inc., Natick, MA).

BrdU administration. The BrdU is an analog of the thymidine that the cell incorporates in the cellular cycle when entering into cellular division. Two hours before the sacrifice of the animals an intraperitoneal pulse of BrdU (60 mg/kg, Sigma-Aldrich) dissolved in physiological saline was administrated to evaluate the proliferative activity of the endogenous stem cells in the SVZ, as well as implanted ASC cells.[23]

At 37 days after stroke surgery, mice in each group were anesthetized with a dose of pentobarbital (60 mg/kg) and fentanyl (0.3 mg/kg) for subsequent intracardiac infusion with 0.9% solution Saline followed by 4% buffered paraformaldehyde (0.1 M phosphate buffer). After perfusion, the brain was removed and washed with 0.1 M PB and cryoprotected by immersion in 30% sucrose. The cuts were performed in a cryostat (model Micron 1800) at 50 microns thick containing the infarct area and the biomaterial implant area.

Histochemistry and immunofluorescence analysis

The sections were washed with PBS, permeabilized with 0.1% Triton X-100 and blocked with 10% normal goat serum. The following primary antibodies were then applied overnight at 4°C: anti-HuNu (Millipore, human cells); GFAP (Dako Cytomation, astrocytes); Anti-CD31 (Abcam, newly formed endothelium); Anti-NueN (Millipore, neurons); Anti-DCX (Santacruz, neuroblasts); Anti-BrdU (Millipore, thymidine analogue); Anti-NF 160 and anti-NF 200 (Millipore, neurofilaments). Subsequently the samples were washed in PBS three times for subsequent incubation in Alexa 488, 555, or 647 (1:500, Invitrogen) conjugated secondary

TABLE I. Counting of Immature (NF 160 kD) and Mature (NF 200 kD) Neurofilaments Found in the Ischemic Zone in Stroke Controls, Stroke Animals After Placement of Biomateriales, and After Placement of Biomaterials with ASC (Units: Neurofilaments Per mm^3)

Group	NF in the Ischemic Zone		NF Into Biomaterial	
	NF 160 kD	NF 200 kD	NF 160 kD	NF 200 kD
Stroke	9.3 ± 6.7	2.1 ± 1.9	–	–
Stroke+ASCs	–	–	36.2 ± 17.9	12.6 ± 9.4
Stroke+BM+ASC	–	–	89.7 ± 13.2	20.4 ± 3.7

Volume analyzed 0.69 mm^3.

antibodies, DAPI-contracted and mounted with the Fluorsave reagent (Calbiochem). Immunofluorescence images were obtained with the Olympus AF2000 confocal microscope.

The quantitative study both at the SVZ and at the areas around the biomaterial consisted in the analysis of 10 different fields for each of the antibodies studied (BrdU, DCX, and GFAP) and the result was the average of 10 determinations. Within the BM, a volumetric analysis was performed using confocal microscopy of 12 optical segments 800 nm thick.

The NF were counted in four optical planes by confocal microscopy in z projection. In cases where the unit of measurement was the observed mark quantity per field (Optical Density, DO), the software program Image J, v1.46r of the National Institutes of Health was used for its calculation.

To estimate the DCX/DAPI index, DAPI-positive and DCX-positive cells were quantified in an area of 300 microns from the ischemic border region at the per-infarct zone. This estimation consists in the number of DCX+ cells divided per the total amount of cells (counted by their nuclei, which is stained with DAPI). Quantification was performed under blind condition.

Statistical analysis

Results are reported as the mean ± SD. Differences between means were determined by Student's t test, with $p < 0.05$ considered significant. One-way analysis of variance followed by the Bonferroni post hoc multiple comparisons test was used to draw comparisons between three groups. We used the program SPSS Statistics 20.0. For the graphical representation of results, the Prism v 5.0 Graph Pad program was used. All data are expressed as mean ± standard error. The graphs of the results were performed using the same program. The criterion for statistical significance was $p < 0.05$.

RESULTS

After 72 h of incubation, cells were easily inserted into the scaffolds, and they were adhered to the walls of the same in a homogeneous way (Figure 1). Karyotype and phenotype did not show any alteration.

The application of $FeCl_3$ induced a limited and constant brain infarction located at the left parietotemporal cortex (Supporting Information Figure 1). Neurological deficits appearared 4 h after the surgery, as forelimb flexion, resistance to lateral push and circling behavior; but all animals returned to a normal neurological examination 12 h later.

Animals did not show any further neurological alteration after the implant of the biomaterials, without inducing or inciting fever or implant rejection, suggesting the absence of pyrogenic elements. Magnetic resonance imaging showed that the implanted biomaterials were fully integrated into the host tissue and reduced the cavitation provoked by stroke in control animals. The reduction was more evident when ASC were added to the biomaterials (Figure 2).

After stroke, there was an astrocytic reaction around the infarcted area. The astrocytic reaction to the biomaterial was not intense (similar to the one produced with stroke alone), but it was much less to the biomaterials precharged with ASC, as we show in Figure 3 (Sham: 0.341 ± 0.028 Stroke: 1.780 ± 0.106; Stroke+BM: 1.681 ± 0.066; Stroke+BM+ASC: 0.935 ± 0.0868).

Neurofilaments were scarcely seen at the infarcted area in control animals, but they were found within the biomaterials. After 30 days of scaffold implantation (with or without ASC) more neurofilaments, both immature (160 kD) and mature (200 kD), were encountered inside the biomaterial

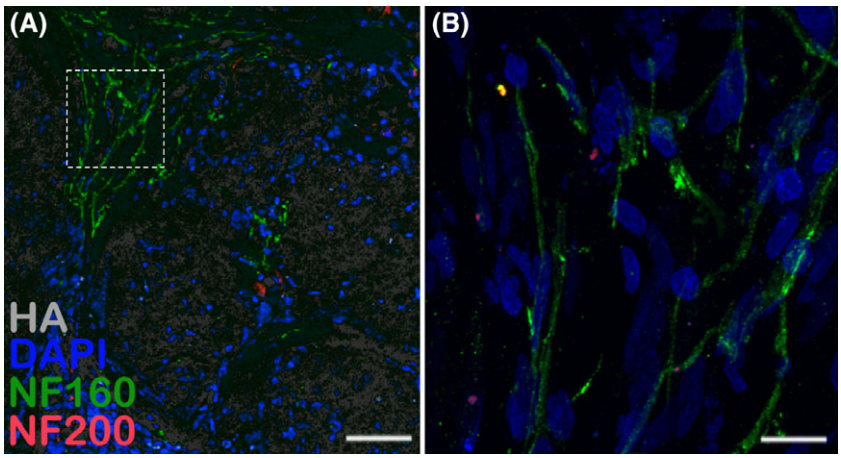

FIGURE 4. Immunohistochemical image showing the neurofilaments found inside the biomaterial+ASC 30 days after implantation. (HA: hyaluronic acid, DAPI: nucleus, NF160: 160 kD neurofilaments, NF200: 200 kD neurofilaments. Scale Bar: 100 μm B: Detail from image A. Scale Bar: 10 μm.

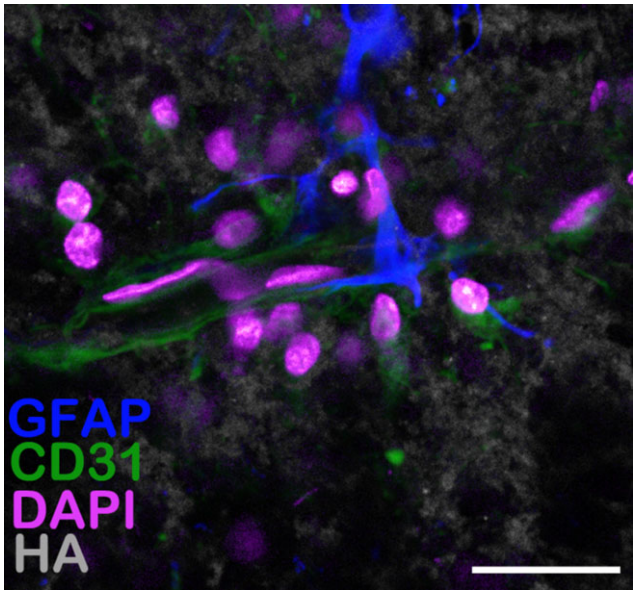

FIGURE 5. Immunohystochemical image showing implanted biomaterials plus ASC, showing CD31 positive cells inside, forming vessel-like structures. Scale Bar: 50 μm.

than in the periphery of the lesion or in the controls (Table I).

More neurofilaments were seen in the biomaterial plus ASC than in the one without them. Most of the neurofilaments were immature (160 kD) (Stroke: 9.3 ± 6.7; Stroke+BM: 36.2 ± 17.9; Stroke+BM+ASC: 89.7 ± 13.2; Table I and Figure 3), but mature neurofilaments (200 kD) were also seen (Stroke: 2.1 ± 1.9; Stroke+BM: 12.6 ± 9.4; Stroke+BM+ASC: 20.4 ± 3.7, data expressed in NF into biomaterial; Table I and Figure 4).

CD31 positive cells were seen inside the implants (Figure 5), but not in the infarcted area in control animals. Some of them were organized in tubule-like structures, resembling vessels. Also, more CD31 positive cells were seen inside the biomaterials pre-charged with ASC.

There was a proliferative reaction at the SVZ after the stroke, in the number of BrdU (Sham: 122 ± 8; Stroke: 174 ± 16; Stroke+BM: 241 ± 11; Stroke+BM+ASC: 305 ± 26) DCX (Sham: 0.51 ± 0.07; Stroke: 0.94 ± 0.08; Stroke+BM: 1.52 ± 0.09; Stroke+BM+ASC: 1.83 ± 0.12) positive cells. The implantation of biomaterials increased this reaction. The preloaded cells (ASC) increased even further this effect (Figure 6).

Newly formed neurons showing DCX were not seen inside the infarcted areas in control animals, although some of them were seen in the vicinity of the core zone. More cells were seen in the vicinity of the scaffold alone and even more around the biomaterials plus ASC. Inside the biomaterials we found some newly formed neuroblasts, and this number increased when ASC were co-grafted (Stroke: 0.032 ± 0.0082; Stroke+BM: 0.061 ± 0.0096; Stroke+BM+ASC: 0.096 ± 0.0169; Figure 7).

Neither HuNu positive cells, not teratomas or any other neoplasic formation were found inside the biomaterials or in the surrounding brain tissue after 30 days of implantation of human ASC.

DISCUSSION

Here, we show that the implant of biomaterials made of HA at the core of a brain stroke lesion permits the migration of endogenous new neurons, glia, and endothelial cells, and the proliferation of neurofilaments and vessel-like structures

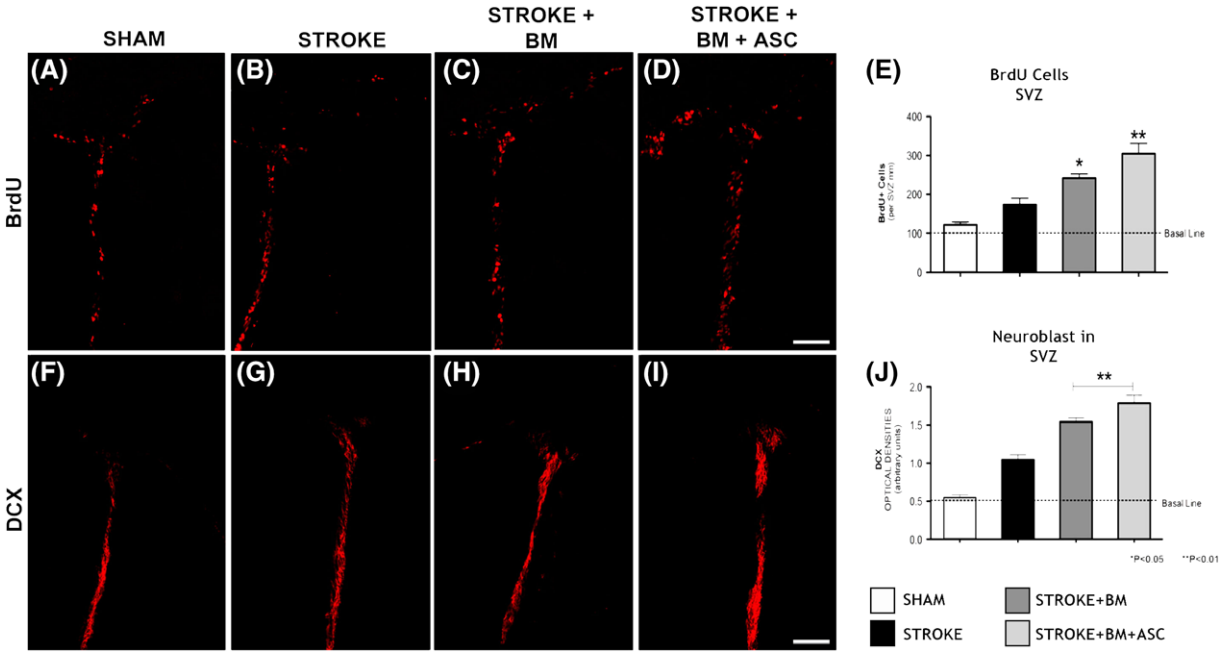

FIGURE 6. Immunohistochemical image showing cell proliferation at the SVZ. A–E: Immunostaining against BrdU. F–J: Immunostaing against doublecortin (DCX) with higher expression of DCX in the SVZ in the experimental group with biomaterials plus ASC. The images correspond the sham group (A and F), stroke (B and G), stroke plus biomaterials (C and H), and stroke plus biomaterials plus adipose stem cells (D and I), and the graph shows the cell count of DCX positive cells. Scale Bar: 200 μm.

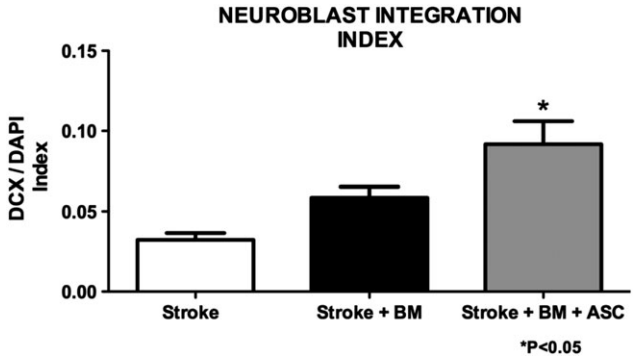

FIGURE 7. Neuroblast integration index. Graph show a data of doblecortin cells express in neuroblast integration index in the vicinity of the lesion. The graph shows a mean ± standard error.

into the biomaterials. Furthermore, the implants increase the proliferation of neuroblasts at the SVZ. The addition of ASCs to the biomaterials increase the proliferative response and the homing effect of biomaterials, plus decreasing the astrocytic reaction to stroke, implantation surgery and biomaterials. It also increases the number of neurofilaments found inside the scaffolds and the number of newly formed vessels inside the biomaterials. The biomaterials are easily integrated into the core zone, and this integration was even better when ASC were associated **(**Supporting Information Figure 2**)**.

ASC has been used to diverse inflammatory pathologies by many authors; being recovery objectives not only in the central nervous system,[25,26] ASC have shown to increase SVZ neurogenesis,[27–31] and have antiinflammatory and cytotactic properties when administered in stroke models,[32,33] and to improve function in animal models, both when administered locally or even through the systemic route.[21,23,34,35] In addition, it has been shown their neuroprotector effect that intervenes directly in the apoptosis mechanism as describe Wei et al.[22,36]; and their secretor effect as show Cunningham et al.[36] However, these approaches do not seem to replace the totally brain tissue lost.[37,38]

Conversely, the use of scaffolds leads to true regeneration or re-formation of new cerebral tissue, not only reducing the bulk cavitation provoked by stroke, but with the presence of new neurons, neurites and supporting cells and vessels, which might functionally integrate with the surrounding brain, as shown Fuhrmann et al., who propose the strategy of favoring the microenviroment.[9,39–41]

Scaffolds made of HA showed compatibility with the brain, as previously noted.[13,14] This confirms the innocuous nature of this biomaterial, which can be designed to fit the particularities of the host tissue, therefore favoring the migration of healthy cells adjoining the lesion zone in several models of injury.[10,42,43] Recently, biomaterials is a growing sector, with very different compositions, such as plasma, PCL, or hydrogels with adhered molecules. All of them share a structural function to restructure the damage tissue.[44–48]

The combination of HA BM+ASC has a synergistic effect, promotes the proliferative activity in the SVZ (increase BrdU and DCX positive cells), enhancing the attraction of endogenous cells, therefore, promoting an appropriate niche to recovery.[49–51] The increase in proliferation of the SVZ analyzed by BrdU agrees with the increase of DCX cells in the group with HA plus ASC. In the group where ASC was not administered, a lower amount of DCX and a greater amount of GFAP is observed. So the administration of ASC and its immunomodulatory or anti-inflammatory effects possibly influence the proliferation and differentiation of GFAP and DCX cells.[23,49–51]

Furthermore, ASC increases the biocompatibility and homing ability of HA scaffolds.[52–54] This is done probably by the antiinflammatory and cytotactic properties of ASC, but not by becoming part of the final implanted structure, since no antigens marking human nuclei were found 30 days after implantation. Also, ASC showed a good integration with HA when the scaffolds were preloaded with these cells, as shown earlier. The combination of the two is safe, as no abnormal cell proliferation of ASC was seen neither in culture nor after implantation. Simultaneously to enhance an appropriate niche and the damage tissue is reduced; the plasticity mechanism is favored.[55–57]

We used immunocompromised animals to explore safety in terms of possible neoplastic proliferation of ASC after implantation.[23,48] However, these cells have not shown rejection issues when used in immunocompetent rodent stroke models.[36,37,58]

In control animals receiving stroke, newly formed neurons (DCX) coming from the ipsilateral SVZ were seen in the vicinity of the core zone, but were not seen inside the infarcted areas. This confirms the observations by others showing that stroke induces per se a repair reaction, but these cells are unable to stay and organize within the core zone.[5,59]

The stroke model we used induced an infarction pattern which is more constant than other models.[60] However, as this model produces few neurological side effects in the animals, we could not observe if this treatment strategy may produce functional improvement.[61–63] Further studies in different models should be done in rodents, or higher mammals should be tested to determine this.

In conclusion, in stroke, the use of HA and ASC is novel. The present results show how scaffolds of HA implanted at the core zone of a stroke lesion permit the proliferation and migration of new neurons, neurites, glia, and vessels, and the addition of ASC increases this proliferation and homing effect **(**Supporting Information Figure 2**)**. This may permit formation of new brain tissue after stroke.

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CONFLICT OF INTEREST

The authors declare no other conflict of interest.

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BRIDGES OF BIOMATERIALS PROMOTE NIGROSTRIATAL PATHWAY REGENERATION

JOURNAL OF BIOMEDICAL MATERIALS RESEARCH PART B

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Bridges of biomaterials promote nigrostriatal pathway regeneration

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Abstract: Repair of central nervous system (CNS) lesions is difficulted by the lack of ability of central axons to regrow, and the blocking by the brain astrocytes to axonal entry. We hypothesized that by using bridges made of porous biomaterial and permissive olfactory ensheathing glia (OEG), we could provide a scaffold to permit restoration of white matter tracts. We implanted porous polycaprolactone (PCL) bridges between the substantia nigra and the striatum in rats, both with and without OEG. We compared the number of tyrosine-hydroxylase positive (TH+) fibers crossing the striatal-graft interface, and the astrocytic and microglial reaction around the grafts, between animals grafted with and without OEG. Although TH+ fibers were found inside the grafts made of PCL alone, there was a greater fiber density inside the graft and at the striatal-graft interface when OEG was cografted. Also, there was less astrocytic and microglial reaction in those animals. These results show that these PCL grafts are able to promote axonal growth along the nigrostriatal pathway, and that cografting of OEG markedly enhances axonal entry inside the grafts, growth within them, and re-entry of axons into the CNS. These results may have implications in the treatment of diseases such as Parkinson's and others associated with lesions of central white matter tracts. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2018.

Key Words: biomaterials, polycaprolactone, ensheathing glia, axonal growth, neurodegenerative diseases

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INTRODUCTION

Damage to the central nervous system (CNS), including stroke, trauma and neurodegenerative diseases, constitute the second cause of death and the first cause of disability worldwide. In order to treat these lesions, regenerative medicine has focused in trying to replace the lost neurons. However, the CNS is a very complex structure, where function depends largely on maintaining long distance point to point connections between the different neuronal nuclei through axons contained in the white matter pathways. The CNS is very unpermissive for axonal regeneration. When a lesion occurs, the basal membrane is rapidly reconstituted and a glial scar is formed. Glial cells segregate inhibitory factors[1] which impede axonal growth.[2-5] This may be one of the causes of the lack of success of cell therapy to treat brain damage. For example, in Parkinson's disease, where dopaminergic cells from the substantia nigra projecting their axons to the striatum are lost, clinical trials have tried to replace the lost dopaminergic cells. Thus, new cells were placed at the striatum, in order to overcome the lack of spontaneous nigrostriatal pathway regeneration. However, the implanted cells were out of control causing the so-called dopaminergic storm, with involuntary movements and other unwanted side effects.[6]

One of the possible solutions for this lack of ability of damaged axons to reestablish long distance connections is to provide of a supporting guide for the axons toward the target structure.[7-11]

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Contrary to what happens at the CNS, axons may grow after being severed within the peripheral nerves. Some approaches to overcome the above problem have used bridges made of peripheral nerve grafts into the CNS to enhance central axonal growth.[12-16] However, issues of availability and histocompatibility of peripheral nerve donors may favor the use of synthetic biomaterials.[9,10] Among them, polycaprolactone (PCL) is an excellent candidate due to its integrative and biocompatibility characteristics.[17,18] It can be used to produce macroporous scaffolds, permitting the outgrowth of axons as well as facilitating the arrival of nutrients, and the disposal of metabolic waste.[19-21] These structures have been used to repair other organs, such as the heart or the urinary bladder.[22,23]

However, re-entry of axons into the CNS is blocked by the presence of astrocytes and the inhibitory factors referred above. The combined use of cells which have been shown to be permissive to this reentry, such as olfactory ensheathing glial (OEG) cells might overcome this problem.[16,24-28]

It has been described that cells from the OEG may have a positive effect in degenerative lesions, promoting axonal growth and regeneration, and even remyelination and synaptic reconnection of the damaged axon.[29,30] This regenerative capacity may be due to the production of trophic factors, like SDF-1 of brain derived neurotrophic factor (BDNF).[31]

In this experiment, we have implanted porous bridges made of PCL, with or without OEG cells embedded inside them, inside the brains of naive rats, between the substantia nigra compacta and the striatum, in order to test the ability of these bridges to convey CNS axonal growth.

MATERIAL AND METHODS

All procedures were performed under the standard criteria for ethical management and care of the animals in accordance with the EC Council Directive of November 24, 1986, and approved by the local ethics committee of the Centro de Investigacion Principe Felipe de Valencia.

OEG isolation and culture

OEG cells were isolated from four adult homozygotic transgenic rats (Wistar-TgN[CAG-GFP]184 years) with constitutive expression of enhanced green fluorescent protein (eGFP) (Rat Resource and Research Center, University of Missouri, according to the procedure described by Navarro et al.[32] Briefly, rats were anesthetized with pentobarbital sodium (60 mg/kg, Sigma) and decapitated. The two outer layers of the olfactory bulb were dissected, minced, and then trypsinized with 0.25% trypsin and 0.03% collagen enzyme IV for 15 min at 37°C. After two centrifugations (1000 rpm, 10 min), the pellet was suspended in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco) with 10% fetal bovine serum (Gibco), 1% glutamine (Hyclone), 2% penicillin–streptomycin (Hyclone), and 1% gentamicin (Hyclone) and seeded into a 60-mm cell culture dish (Corning) with 5×10^6 cells and incubated for 24 h (37°C and 5% CO_2). Cells in passage 4 were prepared to be implanted following the method proposed by the laboratory of Gómez-Pinedo et al.[33]

Polymeric materials

PCL (molecular weight (MW): 48,000, density 1.145 g/cm^3; Polysciences) was employed to develop the porous materials. Poly(ethyl methacrylate) (PEMA) beads (Elvacite 2043; DuPont), having a size of around 200 + 25 μm, were used as the porogen material. Besides, ethanol (EtOH) (+99.5%; Scharlau) and 1,4-dioxane (98% purity, Scharlab) were used in the scaffold fabrication procedure. Each of these materials was employed as received.

Fabrication of scaffolds

A solution of 10% (w/w) of PCL in 1,4-dioxane was mixed with an appropriate quantity of acrylic microspheres in a teflon mold and frozen using liquid nitrogen. The extraction of the frozen mold was carried out in cold ethanol at −20°C (ethanol was changed three times over 3 days) and then porogen was leached using ethanol at 40°C in a shaking bath. Ethanol was changed until no more porogen was detected in the washing solution. Then samples were allowed to dry in air during 24 h and further dried in vacuum for 72 h. Samples were cut using a scalpel to dimensions $1 \times 1 \times 6$ mm and sterilized using γ-ray irradiation at a standard dose of 15 kGy.

Scanning electron microcopy observation

For scaffold morphology characterization, samples were fractured in liquid nitrogen, mounted on copper stubs, and gold sputtered. The samples were observed with a JEOL JSM6300 scanning electron microscope under an acceleration field of 10 kV.

Porosity determination

Porosity was calculated by gravimetric means. Samples were weighed dry, filled with ethanol under vacuum, and subsequently weighed again. Porosity was calculated as the quotient of the volume of pores and the total apparent volume of the scaffold. The volume of pores was calculated as the ratio between the weighed mass of ethanol within the scaffold and ethanol density; the apparent volume occupied by the scaffold was taken as the sum of the pore volume and the polymer volume, and the polymer volume was determined as the ratio of the weighed polymer mass and the density of PCL. Five independent measurements were performed.

The PCL-grafts were stereotactically implanted using a procedure already described.[34] Briefly, animals were anesthetized and placed in a stereotactic instrument permitting trajectories with different entry angles (Stellar, Stereotaxic Instrument 5100, Stoelting Co., IL). Coordinates were determined for the insertion of a graft between the center of the striatum and substantia nigra pars compacta. The coordinates from bregma, according to the Paxinos and Watson[34] atlas, were −1.5 mm lateral (X1), −5.6 mm posterior (Y1), and −8.4 mm below the dural surface (Z1) for the substantia nigra pars compacta, and −4.0 mm lateral (X2), −0.8 mm posterior (Y2), and −5.5. mm dorso-ventral (Z2) for the center of the striatum. The graft's length (or distance between substantia nigra and striatum) measured 6 mm and the entry angles were alpha (cenital) = −28.2° and beta (azimuthal) = 27.5°. A specially designed cannula permitted the placement of the

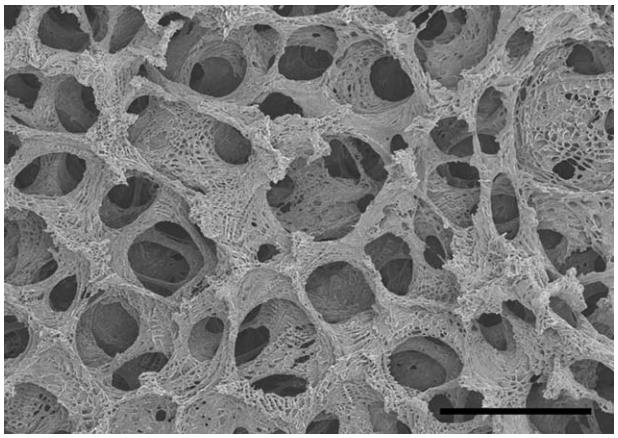

FIGURE 1. Scanning electron image of PCL shows a view of the inner porous structure of the scaffold. It presents a bimodal pore size distribution, with large macropores around 250 μm interconnected and micropores with dimensions ranging from 30 to <5 μm. Porosity of the scaffold as measured by gravimetric means was 90.5 ± 1.7%. Scale bar: 300 μm.

PCL-grafts between the two targets without pushing or squeezing them, with a piston preventing the graft from sliding back. In the experimental group ($n = 8$) we injected at the distal and proximal end of the PCL-grafts 2 μL of a suspension of OEG cells (5×10^4 cells), while in the control group ($n = 8$) the PCL-grafts was implanted without further manipulation. Although the host animals had not been previously manipulated before the implant procedure, the insertion of the PCL-grafts could have produced a local mechanical lesion at the implant trajectory.

Four weeks after grafting, animals were sacrificed through barbiturate overdose, perfused with 4% paraformaldehyde (PFA), removed brains postfixed in 4% PFA solution, and embedded in Tissue-Tek. Transverse sections of 20 μm were cut and mounted on slides. An anti-tyrosine hydroxylase (TH) monoclonal antibody (Millipore MAB318; 1:1000) was used to observe the substantia nigra, and the dopaminergic cells and fibers. An anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (DakoCytomation Z0338; 1:300) was used to analyze the glial reaction, for microglial identification, a monoclonal anti-IBA-1 antiserum (Wako; 1:500) and an anti-green fluorescent protein (GFP) polyclonal antibody (ABCAM AB13970; 1:200), was used to label GFP cells, followed by Alexa secondary antibody 488, 555 and 647 anti-mouse or anti-rabbit.

Immunohistochemistry samples were observed in an optical microscope (Leica DM 6000B, Leica Microsystems) or a confocal microscope (Leica SP2, ABOS Leica Microsystems). To obtain confocal micrographs, each fluorochrome dye within the same field was scanned separately.

Fluorescence images were quantified using the software included in the confocal microscope (Leica SP2, ABOS Leica Microsystems). To measure TH fibers, we applied a modification of the physical dissector method described by Reed, using the unbiased brick principle of the three-dimensional dissector.[35,36]

In order to evaluate the microglial and glial reaction in the area close to the striatal end of the graft (100 μm around), we used a modified version of Sholl rings.[37] Briefly, the stereological graticule consists of concentric circles with 10 μm of distance between each. The cellular somata with their visible branches were placed on the center of the graticule and the number of intersections (NoI) of radial glial cell-like processes projections within the graticule was counted.

Quantitative results are expressed as mean ± standard error of mean. Groups were tested for differences by performing t student test following a Mann Whitney test using Prism (GraphPad Software Inc., La Jolla, CA). Differences were considered statistically significant at a value $p < 0.05$.

RESULT

The biomaterial produced presented the structure of a scaffold with a high porosity. This can be shown in Figure 1, where pores of 300 um are shown beside 5 to 30 μm micropores contributing to connect between them, permitting the passing though of cells and fluids.

All animals survived after the grafting procedure. There were no behavioral or neurologic deficits, and no seizures were observed. The brains showed no macroscopic signs of inflammation, infection or rejection of the biomaterial. Histological analysis with serial sections demonstrated that the graft was correctly placed between the two points chosen and integrated with the host brain. An astrocytic reaction was evident around the graft, without cavitations, edematous or necrotic areas, neutrophil infiltrates or other histologic alterations suggesting rejection. This astroglial reaction was more evident in the grafts implanted without OEG cells, which were encapsulated by the scar and showed protoplasmatic astrocytes. When OEG had been cografted, only a mild astroglial reaction was observed, and the morphology of most of the astrocytes was similar to the intact areas of the brain located far from the implant: non-hypertrophic stellate astrocytes with fine filaments. The quantitative analysis of the astrocytic transformation in terms of number of GFAP marked cells per 500 μm^2 reflects this difference (Figure 2, $p < 0.05$). Figure 2 approximately here.

Also, a discrete microglial reaction in the vicinity of the implanted biomaterial was observed. When OEG was cografted

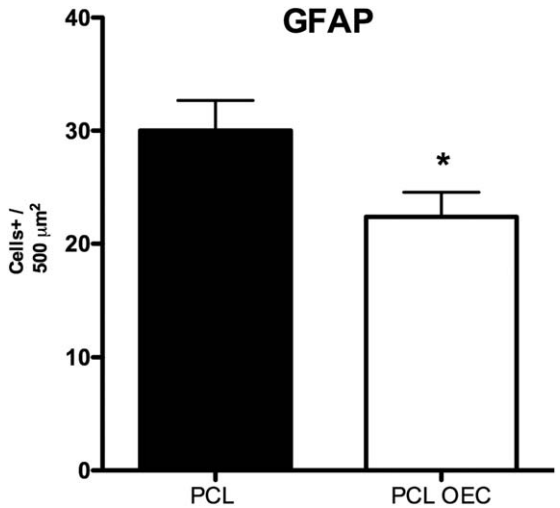

FIGURE 2. Mean density of GFAP marked cells around the implant in both groups. *$p < 0.05$.

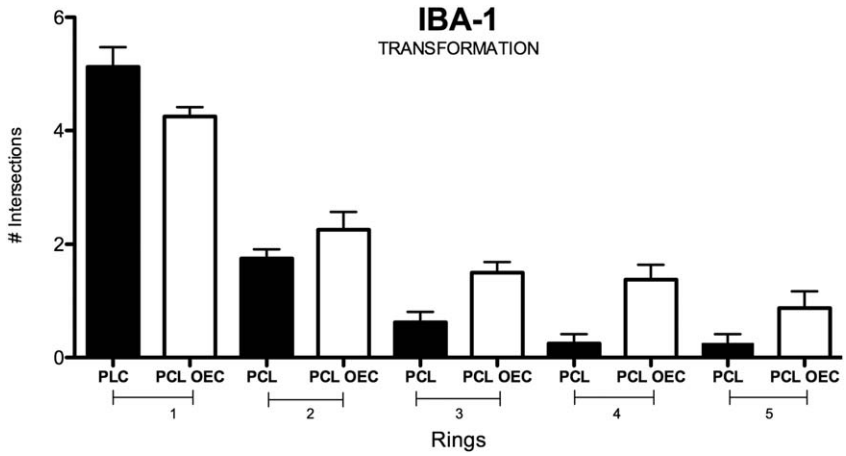

FIGURE 3. Quantification of the microglial transformation around the biomaterial, showing the expression of IBA-1 within concentric rings with 10 μm of distance between each. Expression greater within the innermost rings indicates more ameboid shape, and expression within the outer most, a more branching shape. Microglial cells in resting state tend to be more branching.

the morphology of microglia was similar to other normal brain areas (quiescent microglial cells), while grafts without OEG were surrounded by amoeboid rod-shaped microglial cells forming a small layer around the graft. (Figure 3, $p < 0.05$).

TH+ fibers were seen inside the grafts (in both cases) (Figures 4 and 5), although the fiber density was higher when OEG was cografted (Figures 5 and 6). OEGs were even seen migrating inside the grafts. Fiber count inside the graft showed a higher density at the substantia nigra end, and a progressive reduction of fiber density in the middle and in the striatal end of the graft (Table I, $p < 0.05$).

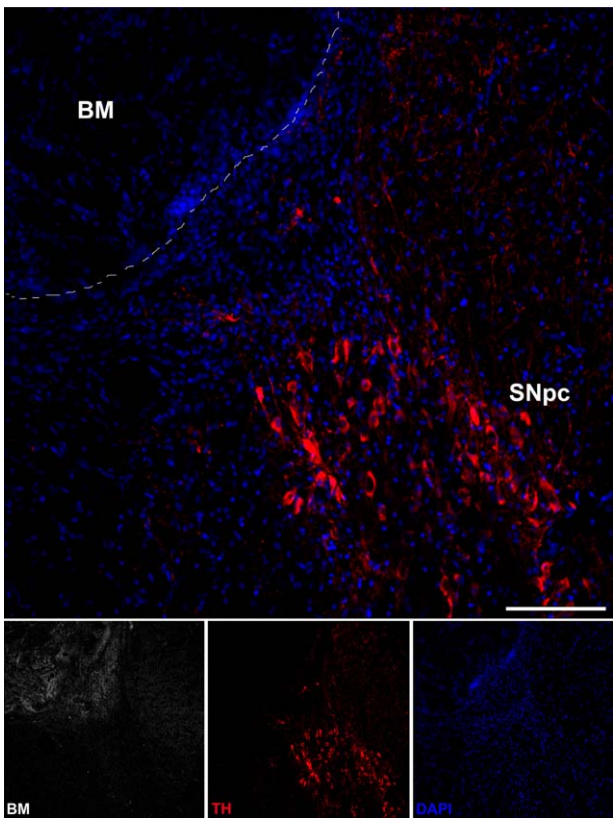

FIGURE 4. Confocal microscopic picture of the PCL only implant close to the substantia nigra pars compacta (SNpc), showing a low density of TH fibers within the biomaterial. BM, biomaterial; TH, tyrosine hydroxylase; DAPI, 4′,6-diamidino-2-phenylindole. Scale bar: 200 μm.

DISCUSSION

Results show that bridges made of PCL support the growth of axons to connect two distant nuclei within the CNS.

Up to now, the only way to produce this was inserting biologically derived materials, either endogenous or allogenic. Peripheral nerve grafts have been widely used due to their capacity to sustain axonal growth. Also, injection of biochemicals to inhibit astrocytic activity and create a local milieu permissive to axonal growth. Collier and Springer[37] were able, using peripheral nerve grafts with embryonic dopaminergic cells in hemiparkinsonian rats, to recover the neurotransmitter levels improving the motor activity of the damaged rats.[38] Wictorin et al. were able to show axons reaching the striatum through Schwann cell grafts in 6OHDA damaged rats, without an effect on motor behavior.[39] The transplantation of fetal dopaminergic neuroblasts in damaged animals may generate a histological pattern resembling the nigrostriatal pathway suggesting a functional integration.

However, up to now no one has demonstrated that the anatomical reconstruction is superior to simply grafting the striatum with dopaminergic neurons. One of the reasons for this may be the limited number of axons reaching their final target. The use of polymeric biomaterials oriented in longitudinal fascicles may be a means to obtain a richer axonal regrowth.

The grafting procedure is original (previously reported in Gómez-Pinedo et al.)[33] and was designed to implant peripheral nerve grafts. It was designed to overcome the difficulty of placing a flaccid material joining two desired nuclei within the CNS. It has been useful to implant biomaterials with the consistency of PCL, the grafts being placed connecting the desired CNS nuclei. The procedure did not produce any observable side effects, in terms of general or neurological behavior, inflammation, infection or rejection.

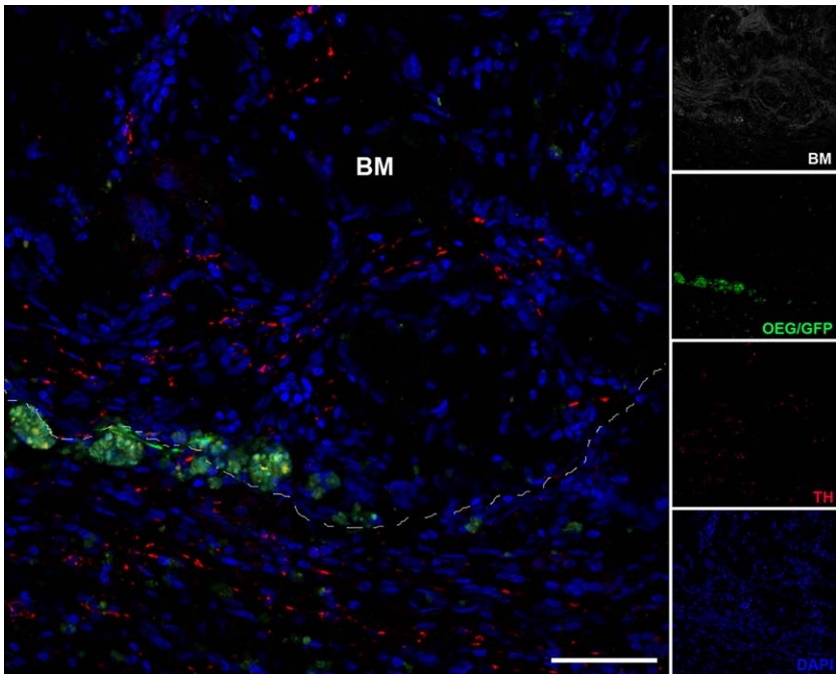

FIGURE 5. Confocal microscopic picture of the PCL plus OEG implant, showing the OEG cells at the border of the biomaterial and a higher TH fiber density within the implant. TH fibers within the biomaterial. GFP, green fluorescent protein; BM, biomaterial; TH, tyrosin hydroxylase; DAPI, *. Scale bar: 75 μm

Also, the PCL graft has shown to be biocompatible with the brain, integrating with the host tissue, not producing toxic side effects, as already described by other authors,[19,21,22] thus able to provide a bridge between the two desired points within the brain, Cografting the OEG cells was associated with a better integration of PCL grafts with the host brain. PCL had already been shown to be permissive to neural growth. In vitro, neural cells grow over the surface of 2D films made of PCL and show a normal electrophysiologic activity.[40,41] PCL has seldom been used *in vivo* in the CNS. Pérez-Garnés et al.[42] showed that PCL can safely be implanted over the meninges, without an inflammatory response. Other biomaterials have been used more frequently in central neural implants, such as hyaluronic acid, chitosan, or carbon nanotubes.[9-11] However, PCL is easily produced, is biocompatible, modulates the microglial and astrocytic response and is permissive for the growth of neurites, although its production is expensive and needs more safety studies in humans. PCL also showed to be compatible to OEG cografting. The use of OEG and other cells which enhance axonal growth associated to biomaterials has already been tested[43,44] and could be a proper strategy to adapt the artificial grafts to the repair of brain injuries.

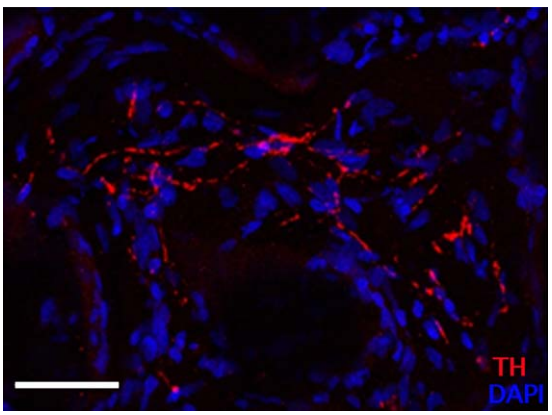

FIGURE 6. Detail of the interior of the PCL plus OEG implant, where TH positive axons are seen within the biomaterial, as well a high density of cells colonizing the implant.

Although there was an astrocytic reaction around the graft, trying to encapsulate the graft, it was mild enough to permit the entry of axons. Also, there was a microglial reaction, indicating a local brain reaction to a foreign body, not important enough to shown signs of graft rejection. Astrocytic and microglial reaction was lower when OEG was cografted, indicating that OEG promotes a microenvironment with less inflammation, which could constitute the mechanism of its enhancing effect on axonal regeneration.

Axons positive to TH+ (i.e., axons from dopaminergic cells) were seen inside the grafts both when PCL was implanted alone and when OEG was cografted. As TH+ fibers have been seen crossing the graft interface at both ends of the grafts, and

TABLE I. Mean Number of TH Fibers Within the PCL Implant in Both Groups[a]

Number of TH+ Fibers		
Zone	PCL	PCL-OECs
SN interface-graft	106 ± 8.2	133 ± 11.1
MW	57 ± 4.8	72 ± 9.9
ST interface–graft	39 ± 4.1	62 ± 3.4

[a] Volume analyzed (0.077 mm^3).

dopaminergic fibers grow only from the SN part of it, it is possible to conclude that the fibers enter the graft at the nigral end, and also that they exit the graft at the striatal end.

This is supported by the fact that fiber density is highest at the nigral end and progressively lower along the direction toward the striatal end. Cografting of cells permissive to fiber reentry into the CNS, such as OEG, markedly increases this effect, being even more marked at the striatal end, that is, it enhances axonal entry inside the graft, growth within the graft and specially reentry of axons back into the CNS. OEGs were seen migrating inside the grafts. The fact that OEG cells have been found along the graft and not only at the extremes, were they were injected, may support the idea that these cells migrate inside the graft and enhance the axonal growth along the graft. They also greatly enhance the reentry of axons inside the brain.

OEGs permit the entry of axons from the olfactory nerves into the olfactory bulb[24-26,45] and have been extensively used a permissive cells for axonal reentry in spinal cord and spinal root repair.[27,33] In our study, OEGs have favored the entry of dopaminergic axons into the graft and they have also permitted the reentry of axons back into the brain.

We chose implanting our grafts close to the substantia nigra pars compacta due to the specific ability of dopaminergic fibers to regrow.[16,46]

This study is limited because we have not shown reinnervation of the tissues from axons coming from the graft. Also, it has to be followed by studies showing histological and functional reinnervation in animal models of axonal injury or neurodegeneration, such as models of parkinsonism, in order to demonstrate the potentially clinical usefulness of this strategy.

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DISCUSIÓN

En este estudio mostramos que la administración de ASCs en la zona de penumbra tras un evento isquémico induce la **proliferación de neuroblastos en la zona subventricular ipsilateral, un aumento de la angiogénesis y tiene efecto antiinflamatorio**. Este efecto se **potencia** sí se administran precargadas en un *scaffold* biocompatible de HA. Además, su administración en el interior de un biomaterial evita que se **difundan** por el parénquima y las mantiene en la diana terapéutica. Por otra parte, también potencia la presencia de neuroblatos en la zona de lesión, células endoteliales y glía, por lo que éste da **soporte estructural** a células sanas que llegan a la lesión, favoreciendo así el nicho y posiblemente, su actividad.

Adicionalmente, mostramos que es posible promover el crecimiento de axones para **conectar dos núcleos distantes** dentro del SNC mediante la implantación de un biomaterial de PCL. Los resultados muestran que los andamios de PCL con células EOGs en su interior **favorecen y guían el crecimiento axonal** no sólo en los extremos del *scaffold*, si no a través de él con mayor eficacia, en comparación con el grupo control.

En el tratamiento del daño cerebral existe la necesidad de promover mecanismos de **reconstrucción**, lo que se traduce en procesos de angiogénesis, neurogénesis, crecimiento axonal, proliferación y migración celular entre otros;[44,92,93] posicionándose así la terapia celular como una opción terapéutica eficaz. Debido a la gran diversidad de tipos celulares aplicables en ésta terapia, es preciso verificar su seguridad y eficacia para cada una de ellas y, de este modo, conseguir su validación como medicamento y su traslado a la clínica. [94]

Dado que la terapia celular implica el implante en el organismo de células vivas, podrían tener cambios comportamentales o funcionales una vez implantadas; además del posible mecanismo de eliminación del organismo, es necesario conocer la seguridad de las mismas. Esto está en contraposición a un fármaco sintético, en el que se conoce tanto su mecanismo de acción como su distribución, metabolismo y excreción.

Sin embargo, en la última década ha habido un número exponencial de ensayos preclínicos con datos de seguridad y efectividad en multitud de modelos patológicos. [95-97] Más concretamente, debido a las particularidades del accidente cerebrovascular, como es la pérdida acentuada de neuronas, astroglía y oligodendrocitos y el insuficiente reemplazo endógeno; la terapia celular se ha perfilado como la terapia restauradora por excelencia. [98,99]

Una de las mayores limitaciones en la terapia de las patologías del SNC es su **accesibilidad a la diana,** ya que el cerebro está recubierto por la BHE que restringe el paso de moléculas.[9] Para ello, es necesario utilizar **vías de administración** que sean capaces de llegar y actúar en la diana terapeútica.

A pesar de que las vías de administración más utilizadas han sido las vías intravenosa, intraarterial e intratecal; la **distribución sistémica** de células ha provocado una acumulación no deseada en pulmones u otros órganos, disminuyendo así su disponibilidad y por tanto, acción en el lugar de la lesión.[100,101] Además, diversos autores[102,103] mantienen que las células que consiguen llegar a la diana, no permanecen más de 2 horas en la misma. Adicionalmente, se ha descrito por Mitkari et al. que la vía intra-arterial no induce angiogénesis[104], y éste es precisamente uno de los mecanismos de las ASCs que más interés tenemos en potenciar. [101]

Por tanto, y con el objetivo de asegurar que el potencial terapeútico del tratamiento actúa en la zona lesionada durante toda la vida útil del implante, hemos elegido la vía de **adminitración local**. Las células son directamente implantadas en la zona de penumbra (vehiculizadas en el *scaffold* o no), facilitándo su acción antiinflamatoria, inmunomoduladora y quimioatrayente.

A pesar de que el acceso directo de un implante celular en la zona dañada parece una alternativa razonable, no existen muchos estudios que hayan abordado esta ruta por los **riesgos invasivos asociados**[105] y la posibilidad de transformación tumoral de las

propias células implantadas.[106,107] En este sentido, nuestros estudios han demostrado tanto la ausencia de formaciones tumorales como de cavitaciones *in vivo*.

Las **ASCs** se han utilizado para **diversas patologías** que cursan con inflamación, no sólo en el SNC. [108,109] Éstas han demostrado propiedades antiinflamatorias, inmunomoduladoras y citotácticas cuando se administran tanto en modelos animales de **lesión isquémica**[110,111] como en ensayos clínicos[112,113], obteniendo resultados que evidencian una recuperación de la función neurológica.[114]

Tras un evento isquémico, se ha descrito la reacción astrocitaria, que se forma alrededor del área infartada aislando la lesión del parénquima. En nuestro estudio, hemos observado una disminución muy significativa en el número de células **GFAP** positivas, sugiriéndo una disminución de astrocitos en el área periinfarto. Además, histológicamente hemos observado no sólo una reducción del número de astrocitos reactivos, si no una **reducción el grosor de la cicatriz glial**. Este patrón, notablemente diferente del grupo control, puede ser atribuído al efecto antiinflamatorio de las ASCs.

Además, hemos mostrado que en los grupos con el biohíbrido (células y biomaterial), hay un menor número de células que expresan **Iba1** en la zona de penumbra, marcador expresado por células de la microglía activadas. Este hecho podría indicar una disminución de la reacción inflamatoria propia de la lesión, reforzándo así, la evidencia de las propiedades antiinflamatorias de las ASCs.

Estos resultados concuerdan con los hallazgos publicados previamente en el estudio de Zhou et al. (2015) en el que mostraron que la administración de ASCs suprimió significativamente la expresión del marcador Iba1 y GFAP comparado con el grupo control.[115] Estos resultados dejarían patente la capacidad de las ASC para disminuir la inflamación a nivel local a través de la supresión de la reacción microglial, y por tanto, mostrando su carácter immunomodulador.[116-117]

Uno de los mecanismos de reparación más relevantes para el evento isquémico es la **angiogénesis**. En dos de nuestros estudios, hemos observado un incremento **células CD31** positivas, en comparación con los animales control. Esto podría indicar un aumento de la vascularización en el grupo tratamiento en la zona adyacente a la lesión.

Estos resultados coinciden los obtenidos en el estudio de Chen y colaboradores, en los que han encontrado que la secreción de diversos fatores angiogénicos, incluyendo VEGF y Angiopoyetina 1 (Ang-1), pormueven la neurovascularización endógena.[118] Es importante destacar que la Ang-1 liberada por las células mesenquimales estabiliza los nuevos vasos sanguíneos formados en respuesta a VEGF; estos vasos se tornan resistentes a fugas y daños causados por la reacción inflamatoria. [119] Adicionalmente, en el estudio de Hao et al. (2013) se encontró que el número de células progenitoras endoteliales marcadas por CXCR4 fue significativamente más elevado en el grupo de ratas tratadas con ASCs; apoyando de nuevo las propiedades angiogénicas de las ASCs.[120]

En la misma línea, hemos observado un mayor número de **células BrdU positivas en SVZ ipsilateral**. Este hallazgo indica un aumento del número de células generadas en el grupo tratado versus el control. Además, y para confirmar el aumento de la proliferación celular en nuestro grupo tratamiento, hemos cuantificado un mayor número de células **DCX positivas en SVZ ipsilateral**, marcador propio de neuroblastos. Estos datos de BrdU y DCX en el grupo control corroboran el ya descrito aumento de la actividad neurogénica *per se* tras un evento isquémico. En esta dirección, en dos de nuestros estudios encontramos un aumento de las **células DCX positivas en la zona periinfarto** asociadas al efecto del tratamiento; pudiendo sugerir la migración de neuroblastos desde el nicho neurogénico. Estos resultados podrían indicar un aumento de la neurogénesis endógena, otro de los mecanismos de las ASCs para mejorar la función neurológica en eventos isquémicos.

En consonancia con nuestros hallazgos, el estudio realizado por Leu y colaboradores mostró un incremento significativo en la expresión de DCX en el área infartada tras la infusión de ASCs por vía intravenosa.[114] En otro estudio reciente,

mostraron que las células madre mesenquimales mejoran la neurogénesis endógena gracias a la expresión del factor neurotrófico derivado del cerebro (BDNF).[121]

El aumento de la neurogénesis endógena podría atribuirse al incremento de la angiogénesis y a la consecuente mejora del flujo sanguíneo cerebral. Por otra parte, la contribución de los factores tróficos no es exluyente, puesto que el BDNF es capaz de estimular la neurogénesis directamente.[122,123] Así mismo, se ha demostrado su efecto **neuroprotector** que interviene directamente en el mecanismo de apoptosis como describe Wei y colaboradores[124], y **secretor**, como describe Cunningham.[125]

Otro de nuestros pilares terapeúticos utilizados son los biomateriales. El uso de andamios en combinación con la terapia celular conduce a una verdadera regeneración, o re-formación del tejido cerebral; no sólo reduciendo la cavitación focal producida por el evento isquémico, sino estableciendo una línea directa de comunicación parénquima sano – biomaterial – lesión. Las células sanas podrían penetrar a más profundidad en la lesión y reconstruir el tejido, disminuyendo el núcleo insalvable isquémico. Como muestran Fuhrmann y colaboradores, el biomaterial favorece éste microentorno. [126-128]

Los andamios de HA mostraron **biocompatibilidad** con el tejido, no habiendo observado ninguna reacción de rechazo al mismo.[83,129] Este hecho confirma la inocuidad del *scaffold*, y su acertado diseño, acorde a las particularidades del tejido huésped. Su forma cilíndrica facilitó su implantación en el tejido, y su composición de HA favoreció su integración en el parénquima, ya que forma parte de la matriz extracelular del SNC. Además, se fabricó con un alto grado de porosidad, para poder albergar células tanto exógenas como endógenas.[77,78]

Nuestros datos se aproximan a los obtenidos en el estudio de Ju et al. en el que el uso de un hidrogel de HA embebido en microesferas de PLGA que contenían VEGF y Ang-1 implantado en el cerebro mejoró el nicho vascular tras una lesión isquémica. [130] Los hallazgos recientes han puesto de manifiesto que el efecto beneficioso de la terapia

celular se ve favorecido por el uso de este tipo de andamios para vehiculizar y mantener en el cerebro los factores terapéuticos.

De igual manera, el biomaterial compuesto de **PCL** ha sido totalmente biocompatible con el tejido, destacando su uso particular para conectar dos regiones distantes. Hasta ahora, la única forma para conectar dos regiones era implantando injertos de nervios periféricos, debido a su capacidad para sostener el crecimiento axonal. Además, era necesario inhibir la actividad astrocitaria para así, crear un medio local permisivo al crecimiento axonal. [131] Nosotros hemos fabricado un biomaterial biocompatible y rígido permitiendo su colocación en el lugar de interés, y de lenta degaradación.[85] Este compuesto ha sido muy utilizado en biomedicina, sin embargo, su uso se ha restringido al sistema nervioso periférico dado sus exitosos resultados en combinación con SC. [132]

Para evidenciar las propiedades de **biocompatibilidad e integración**, se han realizado pruebas de imágen a ratones a término. Es posible observar en estas imágenes de resonancia mágnetica la total integración del biomaterial en el tejido hospedador, asociado a una menor cavitación isquémica.

A pesar de los datos de total integración y biocompatibilidad, observamos una reacción glial del injerto de PCL, tratando de encapsular el injerto, siendo ésta lo suficientemente débil como para permitir la entrada de los axones en el biomaterial. Además, las OEGs disminuyeron en gran medida esta reacción, disminuyendo la reacción inflamatoria.

El **efecto sinérgico** del biomaterial HA + ASCs también es posible observarlo en las imágenes aportadas en el artículo 2. El **volumen de la lesión** es menor en el grupo de animales con el biomaterial sólo, pero mucho menor en el grupo de BM + ASCs, siendo estadísticamente significativa la diferencia del volumen isquémico. El establecimiento de una vía directa de entrada a la lesión, combinado con una matriz en

la que poder asentarse y células exógenas que actúan de bomba trófica, promueve un entorno más favorable para su reconstrucción.[68-72]

Uno de los hándicaps más importantes de la lesión isquémica es el volumen de lesión, la administración de ASCs ha demostrado reducirlo debido a la secrección de factores que promueven la angiogénesis. De esta manera, se consigue restaurar el flujo sanguíneo cerebral y apoyar la neurogénesis endógena. Todos estos mecanismos llevan, en última instancia, a restablecer la función neurológica.[133] Hasta la fecha, distintas publicaciones de ensayos clínicos han mostrado que el transplante de MSCs en pacientes con lesión isquémica reducía el volumen de lesión y promovía la recuperación funcional.[134,135]

Las ASCs mostraron una **buena integración** en el biomaterial de HA cuando fueron precargadas[74], y no se observó proliferación celular anormal ni en cultivo ni después de la implantación.[106,107] Se consigue, simultáneamente, mejorar el nicho adverso y reducir el tejido dañado, facilitándo así los posibles mecanismos de **plasticidad**.

Adicionalmente, hemos cuantificado el número de **Neurofilamentos inmaduros y maduros** (NF 160kD y 200kD respectivamente) en la zona de penumbra en el grupo control y en el interior del biomaterial implantado en el resto de grupos. Se ha observado una diferencia significativa del número de neurofilamentos inmaduros (NF160kD) en el interior del biomaterial sólo versus el grupo control; pero la diferencia se incrementa cuando la comparación es con el grupo del BM+ASCs versus el control. En el caso de los **neurofilamentos maduros (NF200),** hemos encontrado un mayor número de positivos en el grupo BM+ASCs, con respecto al grupo control. Este hallazgo demuestra que los NF son capaces no sólo de sobrevivir en el interior del biomaterial, si no de madurar. Además, es probable que hayamos encontrado un número mayor de neurofilamentos inmaduros que maduros, debido al corto tiempo transcurrido desde el tratamiento hasta el sacrificio como para ver signos de maduración.

Los neurofilamentos son esenciales para establecer una matriz flexible y deformable que soporte el crecimiento axonal; la ausencia de éstos reduce la velocidad de conducción y conduce a alteraciones en el transporte celular. Se ha evidenciado que los niveles de expresión de NF aumentan 7 días después de la isquémia en la zona periinfarto;[136] por tanto, su papel en el escenario post-isquémico es esencial. En el estudio de Gutierrez-Fernández y colaboradores comprobaron que a los 14 días después de la administración de ASCs, los niveles de NF aumentaron signifcativamente.[135,137]

Paralelamente, axones positivos a tirosina hidroxilasa (**TH1**), es decir, axones de neuronas dopaminérgicas, han sido observados en ambos grupos experimentales en el interior del biomaterial de PCL. Este hallazgo sugiere que las fibras TH1+ han podido cruzar la interfaz del injerto desde el extremo situado en la substantia nigra hasta el estriado. Esta hipótesis es apoyada por el hecho de que la densidad de las fibras son mas altas en el extremo nigral, y va progresivamente descenciendo a medida que avanza en la dirección al estriado. La incorporación de células OEGs aumenta esta facilitación de crecimiento axonal en el interior del biomaterial. Se han observado también células OEGs en el interior del andamio, no sólo en sus extremos, avalando la idea de que estas células migran al injerto, se mantienen allí y favorecen el crecimiento axonal en el interior.

La TH es la enzima limitante de la velocidad de biosíntesis de catecolamina, es la encargada de la hidroxilación de tirosina a L-DOPA en las neuronas dopaminérgicas y sus terminales. La evidencia experimental y clínica actual, respalda la hipótesis de que existe una alteración en la neurotransmisión de dopamina después de una lesión cerebral. En un estudio previo realizado por nuestro laboratorio en el que se estudió el potencial de la combinación de OEGs con injertos de nervios periféricos para **promover la regeneración** en la vía nigroestriatal; describieron un mayor número de sinapsis de las fibras TH+ en la vecindad del extremo distal.[132] Estos resultados confirman la capacidad de las células OEG para facilitar la entrada de axones en el SNC, como ya han demostrado en otros estudios.[138,139]

En el estudio realizado por Navarro et al. comprobaron que el transplante de OEGs permite la regeneración axonal pero éstos crecen cerca del transplante[140]; siendo de utilidad en los casos en que las estructuras diana están cerca de la lesión.

El conjunto de estos datos podrían sugerir que tras una lesión cerebral en el sistema nigroestriatal el crecimiento de los axones sea reconducido a través del *scaffold* y que se produzca la inervación dopaminérgica.

La capacidad de colonización de los biomateriales es indiscutible, y su diseño adecuado, no sólo a las características del tejido hospedador, si no a la función que deberá desempeñar una vez implantado, son aspectos fundamentales para desarrollar un tratamiento eficaz.

En ninguno de nuestros estudios hemos encontrado células **Human Nuclei** positivas (un marcador de células humanas), lo que significa que las ASCs no se han integrado en el parénquima cerebral. Lo mismo sucede con las OEGs, que se mantienen en el interior del biomaterial sin llegar a formar parte del tejido. El hecho de que no hayamos evidenciado integración de las células podría indicar que la supervivencia de éstas es agotable y que, por tanto, no permanezcan quiescentes sino que desaparezcan transcurrida su vida útil.[106,107] Dos de los aspectos más importantes a tener en cuenta para garantizar la seguridad de las células es la ausencia de formaciones neoplásicas en el lugar de implantación y su biodistribucción en el organismo. En nuestros estudios no hemos observado formaciones neoplásicas ni cavitaciones en los grupos con ASCs y ASCs + BM.

En este sentido, tanto la seguridad como la efectivad son especialmente relevantes para hacer posible su **traslado a la clínica**. Por tanto, es preciso continuar demostrando la inocuidad del tratamiento mediante histología y pruebas de imagen.[141]

En los estudios de células mesenquimales, se han utilizado animales **inmunocomprometidos** para explorar **parámetros de seguridad**. No han sido encontradas proliferaciones neoplásicas tras la implantación de las ASCs solas, ni precargadas en el biomaterial, ni del propio biomaterial. En paralelo, existen estudios de patología isquémica en modelos de animales **inmucompetentes,** en las que éstas células tampoco han mostrado rechazo o formaciones tumorales. [125,134]

LIMITACIONES METODOLÓGICAS

El **modelo de ictus** que utilizamos induce un patrón isquémico permanente focal y produce reducidos efectos neurológicos en los animales, no habiendo podido demostrar parámetros de mejora funcional. Esta questión, de gran importancia traslacional, debería ser estudiada en profundidad en modelos murinos y mamíferos superiores; mediante técnicas de elecrofisiología y test de comportamiento.[106]

El hecho de que ni las ASCs ni las OEGs sean capaces de **integrarse** en la red nerviosa es una limitación de efecto, ya que su actuación se reduce al periodo de tiempo que sobreviven y permanecen en la diana. Es por ello que la **vía de administración** toma especial relevancia en estos casos. A pesar del carácter invasivo de la administración intracerebral, ésta garantiza que la actuación de la terapia celular se realiza en la zona de interés. La alternativa de vehículizarlas en un *scaffold*, además, las mantiene en la zona del implante sin que se difundan por el parénquima, concentrando aún mas el foco de acción.

Por otro lado, la estrategia terapéutica utilizada repara parte del tejido isquémico, como hemos demostrado anteriormente, pero este enfoque **no parece reemplazar la totalidad** del tejido cerebral perdido. El núcleo isquémico sigue presente y a pesar de los esfuerzos restauradores, hoy en día, los investigadores no han dado con el tratamiento definitivo de reparación total.

PERSPECTIVAS FUTURAS

Para continuar con esta misma linea **de investigación**, lo siguientes pasos se basan en observar y describir procesos de reparación a medio y largo plazo para describir comportamientos de maduración y de re-organización celular. Alargando el tiempo de vida de los animales desde el tratamiento, podríamos evaluar histológicamente si las neuronas inmaduras que han colonizado el biomaterial son capaces de madurar y permanecer en esa localización, y si son capaces de establecer sinápsis y por tanto, restaurar la red nerviosa.

Además, es recomendable estudiar el **grado de recuperación funcional** que genera este biohíbrido, con técnicas electrofisiología y test de comportamiento. Las técnicas comportamentales tienen limitaciones, ya que los roedores tienen una capacidad plástica mayor que la de los humanos, y por tanto, es necesario realizar un alto número pruebas para realmente poder afirmar que exista recuperación funcional. Sin embargo, la electrofisiología es una única técnica, que te permite obtener datos objetivos en un momento puntual. Es posible evaluar su evolución de recuperación a corto, medio y largo plazo, realizándo esta prueba semanalmente y alargando la vida del animal.

En linea con la utilización de ASCs, el uso terapéutico de las vesículas extracelulares excretadas por estas células esta generándo cada vez mas interés en la comunidad científica. Las de un determinado tamaño, son denominadas **exosomas**, y tienen un papel fundamental en la comunicación célula-célula. Las técnicas para su aislamiento se han perfeccionado, pero apenas hay estudios *in vivo* todavía. Al ser moléculas y no células, no tienen capacidad de replicación y por tanto, el riesgo de tumorogénesis se ve aún mas reducido. Además, su tamaño tan súmamente pequeño les confiere una mayor difusión, aspecto que aún se desconoce en su totalidad. En paralelo, muchos autores están investigando el mecanismo de transmisión de información de estas vesículas, ya que hay evidencias de que células endógenas fagocitan estas pequeñas vesículas, pero se desconocen los procesos de señalización una vez en su interior.

En el campo de los **ensayos clínicos**, existen multitud de ensayos con terapia celular basada en células madre mesenquimales de distintos orígenes y utilizando diferentes vías de administración. Hasta ahora y términos generales, la mas utilizada ha sido la intravenosa; sin embargo, debido a los existosos resultados preclínicos en la administración intracerebral obtenidos en el laboratorio adscrito al Servicio de Neurocirugía del Hospital Clínico San Carlos, hay un ensayo clínico en vigor utilizando ASCs por vía de administración local. Las perspectivas de futuro son favorables, ya que en el mismo equipo, estamos trabajando en un borrador para un futuro ensayo clínico de ASCs precargadas en un biomaterial. Es un camino largo, con exigentes validaciones y autorizaciones, pero el esfuerzo se verá recompensado.

En este estudio, hemos demostrado un crecimiento axonal en el interior de un biomaterial de PCL con OECs, sin embargo, el siguiente paso en esta línea es investigar si se han **establecido sinápsis** entre los nuevos axones entre sí, o con otras neuronas. Este campo de reconexión axonal está en expansión y a pesar de los prometedores resultados *in vitro* e *in vivo*, no existen apenasensayos clínicos por el momento.

CONCLUSIONES

Conclusión 1: Las células madre mesenquimales derivadas de tejido adiposo **no producen efectos secundarios en el comportamiento ni en el estudio histológico** al implantarlas en el tejido cerebral difundidas en el parénquima o vehiculizadas en un biomaterial.

Conclusión 2: Las células madre mesenquimales derivadas de tejido adiposo tienen efectos **antiinflamatorios, dismuyendo la reacción glial y la cicatriz astrocitaria**. Además, promueven los procesos **de proliferación en la zona subventricular** ipsilateral.

Conclusión 3: El biomaterial de ácido hialurónico es **biocompatible** y capaz **de integrarse** en el tejido cerebral. Es capaz de actuar como **vehículo** de células exógenas y ser colonizado por precursores neurales.

Conclusión 4: La combinación de un biomaterial de ácido hialurónico con células madre mesenquimales derivadas de tejido adiposo tiene un **efecto modulador,** al reducir **la reacción glial y la cicatriz astrocitaria.** Permite la reparación y reconstrucción del tejido parcialmente dañado, **reduciéndo el volumen isquémico.**

Conclusión 5: El andamio de policaprolactona además de **biocompatible**, **permite el crecimiento axonal** tanto en sus extremos como a través de él, siendo más evidente con la adición de células de la glía olfatoria envolvente en su interior.

CONCLUSIONS

Conclusion 1**:** The mesenchymal stem cells derived from adipose tissue are **do not produce histopathological or behavioral side effects** when implanted in the brain tissue spread in the parenchyma or transported in a biomaterial.

Conclusion 2**:** The mesenchymal stem cells derived from adipose tissue have anti-**inflammatory effects, decreasing the glial reaction and the astrocytic scar**. In addition, they promote **proliferation** processes in the ipsilateral **subventricular zone.**

Conclusion 3: The hyaluronic acid biomaterial is **biocompatible** and **able to integrate** into brain tissue. It is able to act as a **vehicle** for exogenous cells and be colonized by neural precursors.

Conclusion 4: The combination of a hyaluronic acid biomaterial with mesenchymal stem cells derived from adipose tissue has a **modulating effect**, by **reducing the glial reaction and the astrocyte scar**. It allows the repair and reconstruction of partially damaged tissue, **reducing the ischemic volume**.

Conclusion 5: The polycaprolactone scaffold in addition to **biocompatible**, allows **axonal growth** both at its ends and through it, being more evident with the addition of olfactory ensheathing glial cells in its interior.

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